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13. ABSTRACT *(Maximum 200 Words)*

The objective of this project was to develop a system for generating estrogen receptor mutants targeting DNA sequences in estrogen receptor regulated genes in breast cancer cells so that they could be experimentally suppressed ("turned off") or activated ("turned on") in response to small molecules. These estrogen receptor mutants would allow testing of the effect of activating or suppressing specific estrogen regulated genes on the growth of breast cancers. To achieve this objective we set out to develop a novel type of genetically selected mutant estrogen receptor able to bind to the genes of specific estrogen receptor-regulated genes. These mutant receptors could then be converted into chimeric receptors to efficiently and quantitatively suppress both estrogen-dependent and estrogen-independent expression of estrogen-regulated growth stimulatory genes. We used information from a genetic selection done using our modified P222 challenge phage system to identify mutations of interest which were then incorporated into full-length estrogen receptor (ER) for further testing. To repress transcription of estrogen receptor regulated genes we created chimeras in which full length ER, or the ER DNA binding domain, is fused to a powerful KRAB repressor domain. We showed that these chimeras form powerful, easily-regulated, ligand-dependent repressors. Wild-type ER, fused to KRAB domains, was unable to repress transcription of the native pS2 gene, while a KRAB-ER chimera containing a set of up-binding mutations we identified in our challenge phage selections was a powerful ligand-dependent repressor of both basal and estrogen-induced transcription of the pS2 gene. Further analysis of wild and mutant ERs revealed the surprising finding that the affinity of wild type ER for the imperfect ERE half site in the pS2 gene was 100-200 fold lower than its affinity for the consensus ERE half site. Thus, the ability of ER to bind to the pS2 ERE in intact cells largely derives from binding of wild-type ER to the consensus half site and tethering of the second ER monomer to the very low affinity imperfect pS2 half site.

14. SUBJECT TERMS
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Table of Contents

4. INTRODUCTION

Estrogens, act through estrogen receptors. Estrogens play a critical role in regulating the growth and metastases of breast cancers. Growth promotion by estrogen is thought to involve direct estrogen receptor-mediated regulation of the expression of several genes important in cell growth, including those encoding some growth factors (such as TGF-α, IGF1, and their receptors), some early response genes (such as c-myc, and cyclin D1), and other genes, including the progesterone receptor gene. Interference with estrogen activity, usually based on antiestrogens, such as tamoxifen, or on aromatase inhibitors, therefore represents a mainstay in breast cancer treatment. While antiestrogen therapy is often effective initially, the tumors almost always eventually progress to estrogen-independent growth. This limits the long-term utility of endocrine therapies. It is usually accepted that the ability of the 17β-estradiol-ER complex to influence the growth and metastasis of breast cancer cells is due to its ability to regulate the expression of specific genes. The estradiol-ER complex acts directly to induce the expression of a set of "early genes" including c-myc, cyclin D1 and TGF-a, and other genes important in cell growth by directly interacting with these genes. These early genes, and the products of other directly regulated genes, such as the progesterone receptor, may also initiate a regulatory cascade leading to the regulation of downstream genes important in growth control in breast cancer cells. It has been widely proposed that the 17β-estradiol-ER complex complex induces breast cancer cell growth by directly or indirectly regulating the expression of genes important in cell growth control. If genes critical to growth control in breast cancer cells are directly induced by the estradiol-ER complex, then repression of estrogen-dependent and estrogen-independent transcription of these genes should block estrogen stimulated growth of the breast cancer cells. Although it is known that estrogen growth autonomous cells synthesize high levels of growth factors that are normally under estrogen regulation, the hypothesis that the high level expression of growth factor genes is responsible for growth of these cells has never been tested directly. The objective of this work was to develop and use a novel selection system to identify estrogen receptor mutants which exhibit relatively specific ability to bind to and repress (or activate) specific genes estrogen regulated genes important in cell growth and in apoptosis. These targeted repressor (or activator) proteins would allow ligand-regulated control of the expression of key regulatory genes and the assessment of their role in the growth of breast cancer cells.

5. BODY

5A. PROGRESS REPORT

Background.

There are two key features relevant to the development of the repressor proteins.

5A.i. The P22 challenge phage system.

In the bacteriophage P22, the decision between lysis of the infected cells and lysogeny, which allows outgrowth of bacterial colonies is exclusively based on the *Ant* protein, whose production results in lysis of the bacteria. For example, if we generated a recombinant phage with an ERE close to the *Ant* promoter, and a steroid receptor mutant with sufficient affinity binds to this ERE, it will block transcription of the *Ant* gene, producing bacterial colonies. Although conceptually simple, the challenge phage system is technically quite complex, and it required a major development beginning in 1991 for our laboratory to modify it so that it could be used successfully with vertebrate proteins. Because most estrogen regulated genes do not contain the consensus ERE it was necessary to use the imperfect EREs in the target genes of interest. Since effective targeting of an ER mutant to a specific gene cannot be accomplished by binding of ER to the consensus ERE half site (aGGTCA), it was necessary to convert the imperfect half sites in

the genes into palindromes and use these as binding targets in the challenge phage system. For example, to screen for mutants able to bind to the imperfect ERE in the pS2 gene (which is an estrogen-inducible gene often used as a prognostic marker in breast cancer), we converted the native pS2 ERE, 5'-aGGTCActgTGGCCc-3' (the nucleotide which deviates from the consensus sequence is underlined) into a perfect palindrome we call pS2 PAL 5'- GGGCCActgTGGC C C-3' and inserted this sequence into the challenge phage.

5A.ii. The KRAB repressor .

When tethered to a DNA binding domain the KRAB repressor domain, a ~70 amino acid domain found in a substantial number of DNA binding proteins can efficiently suppress transcription of genes containing strong binding site for the protein. The KRAB repressor is functional even at substantial distances from the transcription start site of a gene of interest.

5B. RESEARCH PROGRESS

5B.i. Dimerizing a Genetically Selected Up-binding Mutant-KRAB Chimera with a Flexible Linker results in a Potent Repressor.

To create an effective transcription repressor, we used a flexible linker to artificially dimerize two copies of the genetically selected specificity switch, ERE up-binding mutant, DBD5, and inserted a copy of the powerful KRAB repressor domain at its c-terminus. Repression was determined by the ability to suppress expression of a constitutively active promoter containing EREs. The linker-dimerized DBD5-KRAB (dDBD5K) chimera is a much more potent repressor of transcription of an ERE-containing gene than the DBD5-KRAB (DBD5K) monomer. DBD5K did not repress expression of the native pS2 promoter, while dDBD5K elicited a dose-dependent repression of both constitutive and estrogen-receptor induced expression of the pS2 test promoter. The linker dimerized protein dDBD5K is specific for genes containing the ERE, and does not repress transcription of a test gene containing the glucocorticoid/progesterone response element (GRE/PRE).
Artificially dimerizing the genetically selected DBDs using a flexible linker results in a dramatic increase in their potency as sequence-specific transcription repressors. In addition, artificially dimerizing the genetically selected mutants allows for the covalent joining of two different proteins, each targeted to a specific DNA sequence. This will greatly increase the specificity and potency of the targeted mutants.

5B.ii Ligand-Regulated Repressor Chimeras.

We append copies of one paper describing the development of our novel repressor proteins which was published this year in the *Journal of Biological Chemistry*. A second manuscript is in preparation, and is summarized below. A key finding in our early work was that a KRAB-ER-KRAB chimera, which efficiently repressed transcription of synthetic genes containing multiple EREs, was unable to repress expression of the native pS2 gene, which contains a single imperfect ERE. Thus, even though transcription of the pS2 gene is induced by estradiol-ER in intact cells, the KRAB-ER-KRAB chimer is unable to suppress transcription of this gene. Our data is consistent with the view that while activation of transcription may only require transient association of a steroid receptor with the DNA, repression requires a more continuous association. This data indicated that only by using mutants which bind and target the imperfect ERE in the gene of interest could we suppress its expression. An ER-KRAB chimera (KERK-3M), into which we inserted a set of mutations we identified in a challenge phage selection, was a potent repressor of both basal and estrogen-dependent expression of the pS2 gene.

5B. iii. Interaction of pS2 Binding ER mutants with pS2 EREs.

Our work on the development of mutant ERs able to bind with high affinity and specificity to the pS2 ERE indicated that selection of such mutants was extremely difficult. This was initially

surprising since the pS2 ERE is clearly activated by wild-type ER in intact cells and differs from the consensus ERE by only one nucleotide (ps2 ERE: 5'-aGGTCActgTGGCCc-3'; Consensus ERE: 5'-aGGTCActgTGACCt-3'). To analyze the interactions of our mutants with the consensus ERE (cERE), with the native pS2 ERE and with the imperfect half site converted into a palindrome (ps2 PAL: GGGCCActgTGGC C C-3'), we prepared a series of ER mutants identified by a combination of genetic selection and modeling as likely to bind to the native pS2 ERE. These mutants were expressed in transiently transfected mammalian cells, and extracts were prepared. In order to ensure that we used the same amount of wild type ER and of each ER mutant, the levels of ER in the samples were determined by Western blotting (Fig. 1, panel A). We analyzed the ability of these mutants to bind to all three of the EREs *in vitro* using protein titrations in gel mobility shift assays.

The wild type ER bound to the cERE with higher affinity than any of the mutants (Fig. 1, panel B). Most of the mutants bound to the ERE in the pS2 gene with a significantly higher affinity than the wild type ER, which bound moderately well (Fig. 1, panel C) . Interestingly, the 3M mutant used in our earlier work bound with a higher affinity than the wild-type ER, but was not the highest affinity mutant. When we looked at the ability of wild-type ER and of each of the mutants to bind to the imperfect ERE half site in the pS2 gene converted into a palindrome (PS2 PAL), the results were quite surprising. While the ER mutants showed clearly detectable binding to PS2 PAL, their affinity for this ERE was in all cases is ~8-10 fold lower than the affinity of wild type ER for the cERE (Fig. 1, panel D). In other words ~10 μg of extract expressing the ER mutants was required to shift 50% of the PS2 PAL probe while only about 1.5 μg of extract expressing the wild-type ER was required to shift 50% of the cERE (Fig. 1 panels B and D). The wild-type ER had barely detectable ability to bind to the palindrome containing two copies of the imperfect ERE half site in the pS2 gene (PS2 PAL). We estimate that the affinity of wild-type ER for the PS2 PAL is 100-200 fold lower than its affinity for the cERE (Fig. 1, panel D). This important new finding means that the ability of wild-type ER to interact with many ER-regulated genes which contain an imperfect ERE half site and a consensus half site is almost completely based on binding of one monomer of the ER dimer to the consensus half site and tethering of the other monomer of the ER dimer to the imperfect half site to which it binds with very low affinity. This is quite different than previous views of the binding of the ER to imperfect EREs which led most researchers to assert that the affinity of the ER for imperfect ERE half sites in most genes was 2-5 lower than its affinity for the cERE half site.

We also prepared a palindrome containing the imperfect ERE half site in the Bcl-2 gene and the imperfect ERE reported in the TGFα1 gene. The wild-type ER showed no detectable ability to bind to these sequences in gel shift assays at any level of ER tested (data not shown). Based on the sensitivity of our assays, we conclude that wild-type ER binds to each of these EREs with an affinity >250 fold lower than its affinity for the cERE.

We next carried out transient transfections to assess whether the *in vivo* ability of wild-type ER and of the ER mutants to activate transcription was proportional to their ability to bind to the EREs. Since the binding of wild-type ER to the PS2 PAL was so poor, we constructed reporter genes containing two copies of each of the elements. Activation of transcription on the cERE was most efficient with the wild-type ER. The ER mutants activated transcription from the cERE less effectively than wild-type ER (Fig. 2, top panel). Several of the ER mutants were more effective in activating transcription from the pS2 ERE than wild-type ER (Fig. 2 , middle panel, mutants 3M, 4.3 and 4). Wild-type ER was unable to activate transcription from PS2 PAL, while the mutants showed clearly detectable ability to activate transcription. (Mutant 6.2 which does not activate transcription was the only mutant to show very low binding to the pS2 ERE and no detectable binding to PS2 PAL. These data indicate that the ability of the wild-type ER and of the ER mutants to activate transcription in intact cells was roughly proportionally to their ability to bind to the various EREs *in vitro* in gel mobility shift assays.

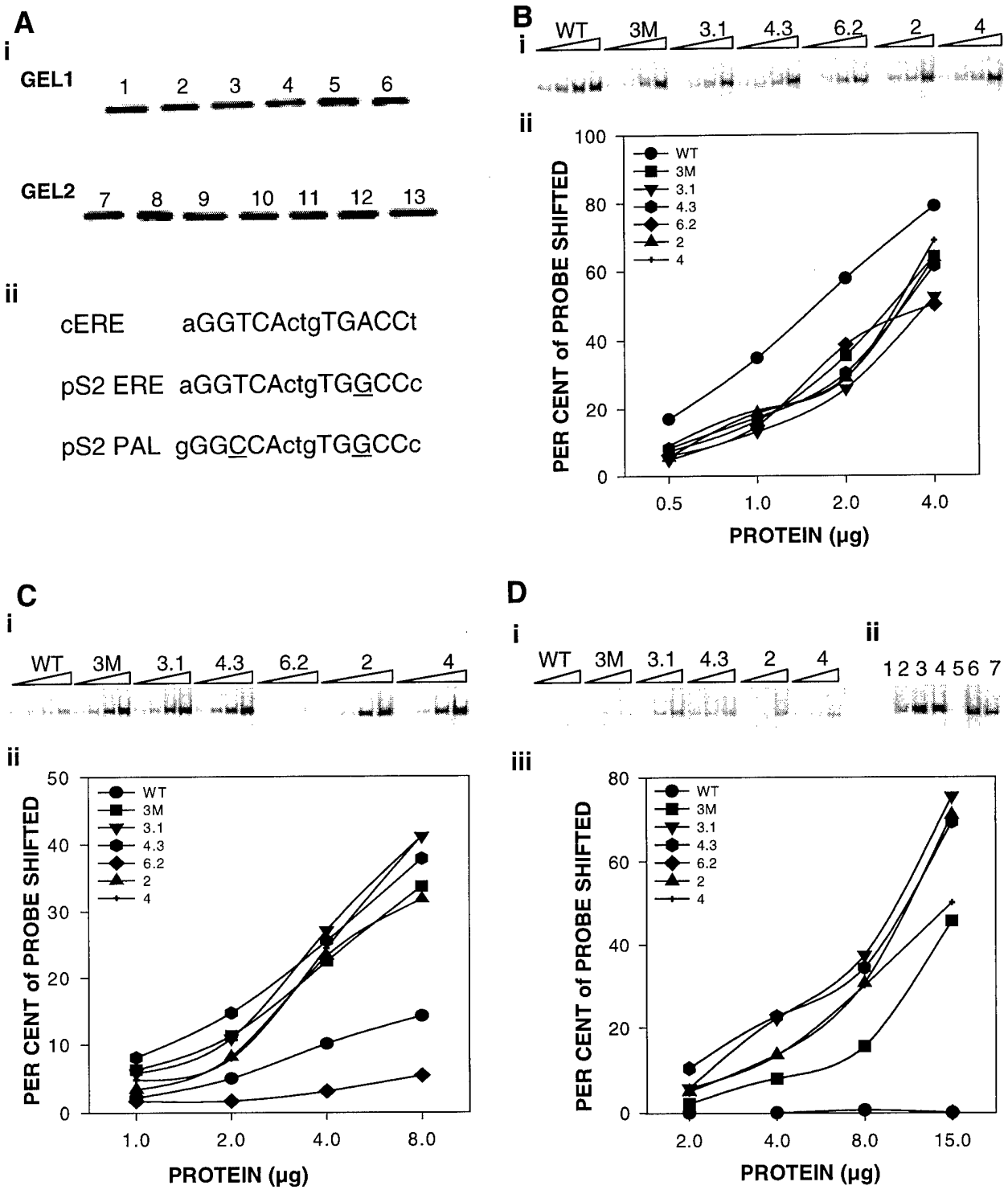

Fig.1. Binding of estrogen receptor mutants to the consensus ERE and to pS2-related EREs. The legend for figure 1 is on the next page.

Fig. 1. Binding of estrogen receptor mutants to the consensus ERE and to pS2-related EREs. (Figure legend). Wild-type ER and the several ER mutants were expressed by transient transfection in CHO-S cells (Life Technology), crude extracts (high speed supernatants of salt extracted cells) were prepared, Western blotting with an anti ER monoclonal antibody was carried out and the extracts were normalized so that each reaction contained an equivalent amount of ER, or of the indicated ER mutant. The ability of the wild-type ER or the ER mutants to bind to the consensus ERE, the ERE in the native pS2 gene, and a palindrome derived from the imperfect ERE in the pS2 gene was evaluated in electrophoretic mobility shift assays carried out across a range of protein concentrations. Panel A.i. is a Western blot showing that we have successfully normalized the samples so that we are delivering an equivalent amount of ER in each reaction. Differences in expression levels were small and normalization required only minor adjustment in sample volumes. The nucleotide sequences of the three test EREs, the cERE, the pS2 ERE, which is the ERE in the native pS2 gene, and the pS2 PAL, which is the imperfect half site on the right side of the pS2 ERE converted into a palindrome are shown in panel A.ii. For each gel shift assay in panels B-D the raw data showing the gel shifted bands is shown on top and a graph showing the per cent of the labeled ERE probe whose mobility is shifted is shown below. Panel B shows the binding of the wild-type ER and the ER mutants to the cERE. The wild-type ER (filled circles) shows higher affinity binding as judged from the smaller quantity of extract required to up-shift 50% of the labeled cERE probe. The ER mutants showed similar binding to the cERE. Panel C shows binding of the ER mtuants and the wild type ER to the ERE in the native pS2 gene (pS2 ERE) Most of the mutants show similar binding to the pS2 ERE, and this is substantially higher affinity binding to the ERE than wild-type ER. Not all mutants bind better to the pS2 ERE than wild-type ER. Mutant 6.2 (filled diamond) shows similar binding to the cERE as the other mutants, but did not bind well to the pS2 ERE. Panel D shows binding of the ER mutants and of wild-type ER to the pS2 PAL. Because higher protein concentrations were required for binding, the data for 15 mg of extract protein in panel D.ii. Wild-type ER (filled circles) and mutant 6.2 (filled diamonds, not visible under the filled circles) showed very little ability to bind to the pS2 PAL. Binding to the pS2 PAL by wild-type ER was not truly zero as it was detectable, while binding to the TGFα1 and BCL-2 EREs was undetectable (data not shown). There were some differences detected in the affinity of the mutants for the pS2 PAL. The amount of protein required to up-shift 50% of the pS2 PAL, ~10 μg, is far higher thanthe ~1.5 μg, required for the wild-type ER to up-sift 50% of the cERE (C. Mao and D. Shapiro, unpublished observations).

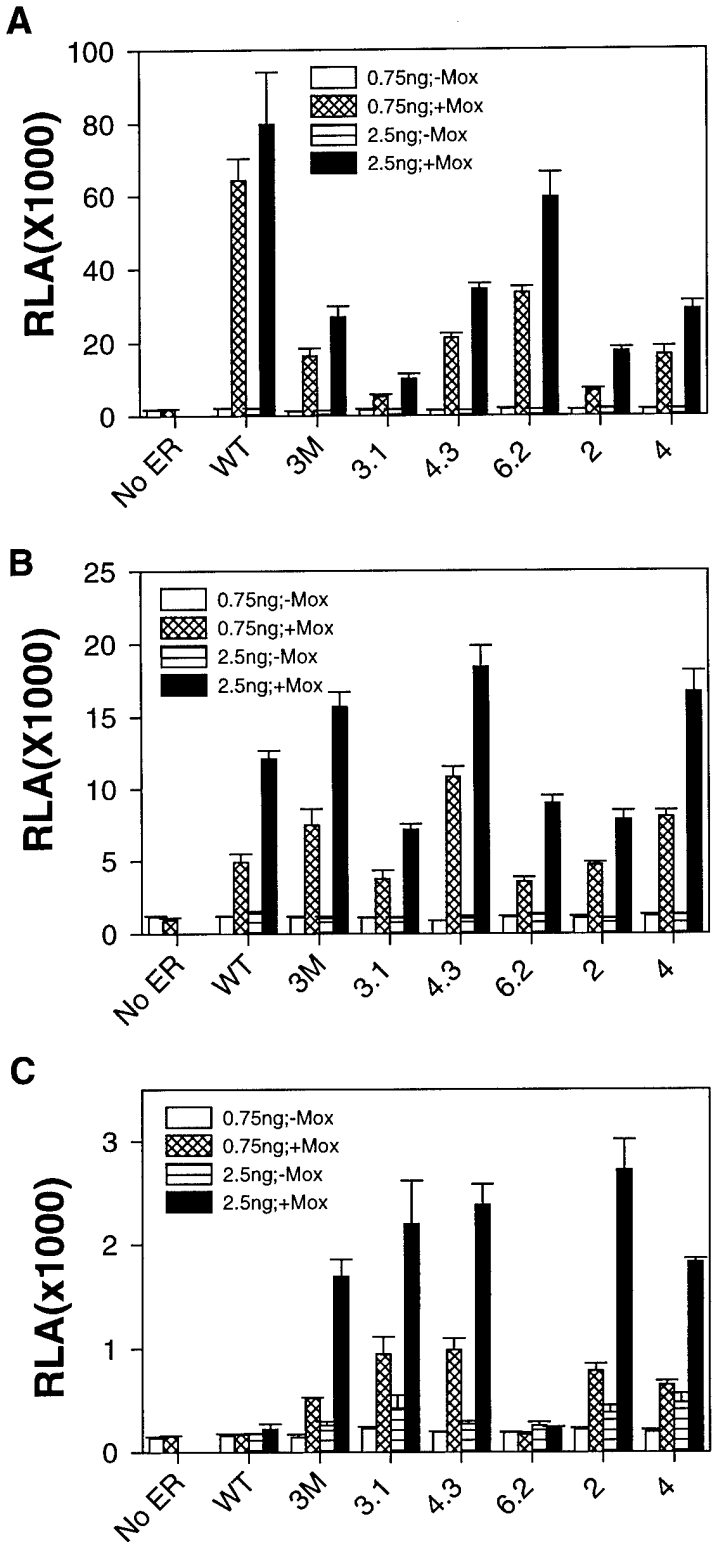

Fig.2. Transactivation by ER mutants is related to their ability to bind to the consensus ERE and to pS2-derived EREs. The legend for figure 2 is on the next page.

Fig. 2. Transactivation by ER mutants is related to their ability to bind to the consensus ERE and to pS2-derived EREs. (Figure legend). Because the wild-type ER bound so poorly to the pS2 PAL, we constructed test genes containing two copies of each ERE upstream of a consensus TATA box and driving transcription of a luciferase reporter gene. For each transfection the indicated amounts (0.75 ng or 2.5 ng) of an expression plasmid driving production of the wild-type ER or one of the ER mutants was co-transfected into the cells. Transfections and luciferase assays were performed as described in the appended paper (De Haan *et al*,). Panel A shows transactivation of a cERE-containing reporter gene. Transactivation was ligand-dependent in all cases and the wild-type ER was the most potent transactivator. Panel B shows transactivation of the ERE in the native pS2 gene (see Fig. 1A. ii.) Transactivation was dependent on the presence of an estrogenic ligand. Several of the mutants which showed higher affinity for the pS2 ERE than wild-type ER in the gel mobility shift assay (Fig. 1C), showed higher levels of transactivation than wild-type ER. Most striking was panel C. Wild-type ER and ER mutant 6.2 which showed very little ability to bind to the pS2 PAL in gel mobility shift assays, showed essentially no ability to transactivate the pS2 PAL reporter in transfections of intact cells. In contrast, the ER mutants which bound to the pS2 PAL in gel shift assays (Fig. 1D) showed clear ligand-dependent transactivation of the pS2 PAL in intact cells. Consistent with the much lower affinity of the mutant ERs for the pS2 PAL (compare Fig. 1B and Fig, 1D) transactivation from the pS2 PAL was many fold weaker than from the cERE. These data show that there was a generally good correlation between the ability of an ER to bind to an ERE *in vitro* in gel mobility shift assays and its ability to activate transcription from a the ERE in intact cells. The data also show that our genetic selection was absolutely necessary to obtain interaction of an ER with the imperfect ERE half site in the pS2 gene (C. Mao and D. Shapiro, unpublished observations) .

While these data showed that we could effectively target the pS2 ERE, they revealed an important issue which has occupied much of our time in the past year. When we began work with the challenge phage system, we were operating under the view (derived from substantial indirect work over the years in many laboratories) that the affinity of the ER for imperfect ERE half sites was a few fold less than its affinity for the consensus ERE half site. However, our direct binding studies indicated that the wild-type ER binds to the imperfect half site in the pS2 gene >100 fold less well than to the cERE half site. In addition, there was no detectable binding to the imperfect half sites in the Bcl-2 and TGFα1 genes. This poses a major challenge in use of the challenge phage system. In the bacteriophage P22 challenge phage system, the signal to noise ratio (ie. The number of false positive clones), is less than 1 per million, and we showed that a single positive DNA can easily be detected among a million negative DNAs (see Appended paper Table 1). This exceptional selectivity is a major strength of the challenge phage system, and is not shared by other selective systems, such as phage display or yeast display. This low level of false positive colonies is achieved because only proteins which bind with a reasonably high affinity to the ERE inserted into the phage can disrupt Ant expression blocking lysis of the bacteria and resulting in formation of bacterial colonies. Mutant proteins with a low affinity for the ERE will not prevent lysis and are scored as negative in this assay. When we create a large pool of receptor mutants, if mutants with an affinity for the imperfect ERE palindrome of interest a few fold greater than the starting wild-type ER DBD are all that is required for colony formation, than these mutants will be reasonable rare, but still easily detected in the challenge phage system. However, since the affinity of the wild-type ER for the sequences in imperfect ERE half sites is extremely low, only those mutants exhibiting very large increases in affinity for the imperfect ERE will be identified as colonies. The number of mutants which in a single cycle of mutation and selection will have the requisite 10-100 fold increases in affinity will likely be extremely rare. In fact, we did not identify any positive colonies in our first screening using the either of the 2 TGFα1 imperfect EREs or the imperfect Bcl-2 ERE. Since the wild-type ER shows no detectable binding to these imperfect EREs, extremely rare mutants with a very large increase in affinity would have to be produced. Historically this class of mutants has most often been produced as a consequence of a labor intensive strategy using several small selection steps, rather than by screening of a single mutant pool. Since this strategy of stepwise selection for small incremental increases in affinity cannot be used with the p22 challenge phage system, and we lack the personnel and funding to implement a new strategy based on multiple screening cycles, we have focused much recent effort on increasing the throughput in the challenge phage system. While our original screen involved only 5,000-10,000 colonies/plate (see appended reprint) by a series of modifications in the methods for the production of the mutant library, in the transformation and outgrowth system, we can now screen almost a million colonies/plate. With this high level of throughput it is at least potentially possible to identify extremely rare mutants with the requisite binding affinity for imperfect EREs.

5C. Progress on Statement of Work.

Task 1: Months 1-12: <u>We will prepare potent repressors of estrogen regulated genes (ERG-repressors)</u>.

Progress: Potent repressors of one estrogen regulated gene (pS2) were prepared and characterized and a paper based on that work has been published in *J. Biol. Chem.*.

Task 2: Months 6-18: <u>We will characterize the ability of the ERG-repressors to suppress transcription of synthetic reporter genes and of endogenous ER-regulated cellular genes</u>.

Progress: A pS2 repressor has been characterized and much of the characterization is included in the paper in *J. Biol. Chem.*. Additional ER mutants, which bind to the pS2 ERE have been identified and their characterization is described here (Figs. 1 and 2). Some of these mutants

exhibit a slightly higher affinity for the pS2 ERE than our previously described 3M mutant. A manuscript describing this work will be prepared in the next few months.

Task 3: Months 16-30: We will construct stable cell lines expressing the ERG-repressor.

Progress: In another project, we recently described a novel way to prepare stable cell lines expressing toxic proteins (Zhang, C.C. *et al.*, 1999. *Mol. Endocrinol.*, 13, 632-643). However, since abrogating expression of the pS2 gene has no obvious effect on the growth of breast cancer cells, the preparation of stable cell lines was a lower priority than efforts to obtain repressors targeted to more critical genes.

Task 4: Months 18-36: We will test the ability of the ERG-repressor to suppress the growth of ER^+ MCF-7 cell lines (parental, and antiestrogen resistant, and estrogen-autonomous) and of ER^- 231 cells.

Progress: Since the only gene we specifically targeted was a prognostic martker, pS2, it would not have been possible to block growth of the cells by abrogating its expression.

Task 5: Months 24-36: We will evaluate the ability of the ERG-repressor to block the growth of solid tumors derived from antiestrogen resistant and estrogen autonomous MCF-7 cells and from 231 cells.

Progress: These studies would only have been appropriate if the powerful repressors able to block the growth of breast cancer cells *in vitro* had been prepared. We therefore focused our limited resources on developing the challenge phage system to make possible future isolation of such targeted repressors.

6. KEY RESEARCH ACCOMPLISHMENTS.

- We developed a novel selection system for identifying mutated proteins targeted to genes of interests and used this system to identify a novel set of ligand-dependent chimeric proteins which repress the expression of genes containing estrogen response elements.

- We demonstrated that flexible linkers creating chimeric ER DBDs can be used to enhance the ability of mutated ER DNA binding domains to dimerize and repress transcription from ERE-containing reporter genes

- We demonstrated repression of basal and estrogen induced expression of the native pS2 gene, a prognostic indicator in breast cancer.

- Insertion into the chimeras of mutations we identified by our genetic selection as enhancing binding to the estrogen response element was critical was critical for achieving repression of the pS2 gene.

- We showed that wild-type ER exhibits very low affinity for the imperfect ERE half sites in estrogen receptor regulated genes. This leads to the new concept that the ability of ER to activate transcription from these genes is largely based on binding of one monomer of the ER dimer to the consensus half site and weak tethering of the other ER monomer to the imperfect half site.

Most Important Problem

- We find that the ER has little or no ability to bind to the imperfect ERE half sites in the EREs of several genes of interest. This means that we must identify very rare mutants exhibiting large order of magnitude increases in affinity for these EREs using our challenge phage system. This required major changes in the selection system to increase its throughput. In consequence, the effort required to select, characterize and introduce into breast cancer cells repressors to several different ER-regulated genes requires multiple researchers, and is far beyond the quite limited resources provided for this project.

7. REPORTABLE OUTCOMES

7A. Journal Articles

Chusacultanachai, S., Glenn, K.A., Rodriguez, A.O., Read, E.K., Gardner, J.F., Katzenellenbogen, B.S. and Shapiro, D.J. (1999) Analysis of estrogen response element binding by genetically selected steroid receptor DNA binding domain mutants exhibiting altered specificity and affinity. *J. Biol. Chem.*, **274**: 23591-23598.

De Haan, G., Chusacultanachai, S., Mao, C., Katzenellenbogen, B.S. and Shapiro, D.J. (2000) Estrogen receptor-KRAB chimeras are potent ligand-dependent repressors of estrogen-regulated gene expression. *J. Biol. Chem.*,**275**: 13493-13501.

7B. Abstracts. And Presentations.

Shapiro, D.J. USAMRMC Breast Cancer Research Program Meeting. "Era of Hope" Atlanta, GA, June 2000.

7C. Patents and Licenses.

None

7D. Degrees Obtained Supported by this Award.

Georgius de Haan, Ph. D., 2000 (supplies only)
Sudsanguan Chusacultanachai, Ph.D. 1999supplies only)

8. Conclusions.

The feasibility of producing small molecule-regulated repressor proteins targeted to a specific estrogen receptor regulated gene has been demonstrated. A mutant estrogen receptor KRAB repressor chimera was developed which effectively targeted the pS2 gene (often used as a prognostic marker in breast cancer) for repression. The finding that estrogen receptor binds with little or no affinity to the imperfect estrogen receptor binding sites in several important estrogen receptor regulated genes was surprising. This finding changes the way we look at interaction of estrogen receptor with the imperfect estrogen response elements in estrogen regulated genes, and explains why it is so difficult to produce these gene targeted mutants. Several aspects of the challenge phage selection system have been optimized to allow screening of the very large numbers of mutants which will be required if mutants targeted to a specific gene are to be identified in future studies. Future efforts to target and repress or activate specific genes using this system will require a larger effort in personnel and support than is possible using the present funding system.

9. References.

None included

10. Appendices

a. 3 Copies of 2 Published papers

12. List of Individuals Receiving Salary Support from DAMD 17-97-1-7241

David Shapiro, Professor, Principal Investigator
Chengcheng Zhang, postdoctoral
Chengjian Mao, postdoctoral
Monica Monatano, postdoctoral
Kirk Ekena, postdoctoral
Seongeun Park, graduate student
Edmund Chang, graduate student
Sijun Zhu, graduate student
William Harrington, graduate student
Claire Pollard, undergraduate hourly
Francesca Antonnachi, undergraduate hourly

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Estrogen Receptor-KRAB Chimeras Are Potent Ligand-dependent Repressors of Estrogen-regulated Gene Expression*

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As an approach to targeted repression of genes of interest, we describe the development of human estrogen receptor (ER) α-KRAB repressor domain chimeras that are potent ligand-dependent repressors of the transcription of estrogen response element (ERE)-containing promoters and analyze their mechanisms of action. Repression by the KRAB domain was dominant over transactivation mediated by ER AF1 and AF2. An ERE and an ER ligand (estrogen or antiestrogen) were required for repression. Studies with several promoters and cell lines demonstrated that the presence of EREs, rather than the capacity for estrogen induction, determines the potential for repression of a gene by the KRAB-ERα-KRAB (KERK) chimera. A single consensus ERE was sufficient for repression, but the KERK chimera was unable to suppress transcription from the imperfect ERE in the native pS2 promoter. We recently reported mutations that enhance binding of a steroid receptor DNA-binding domain to the ERE. Introducing these mutations into wild-type ER enhanced transactivation from the pS2 ERE. Insertion of these mutations into KERK created the novel repressor KERK-3M, which is a potent repressor of both ER-induced and basal transcription on a promoter containing the pS2 ERE. These modified ER-KRAB chimeras should prove useful as new tools for the functional analysis and repression of ER-regulated genes.

Generating ligand-regulated activators or repressors targeted to DNA sequences in any gene of interest represents a challenging long-term goal of protein engineering. The model systems we use to approach this objective are based on estrogen-regulated genes. The effects of estrogen are mediated by the estrogen receptors ERα[1] and ERβ. ERs are ligand-activated transcription regulators that are capable of high affinity binding to a specific DNA sequence, termed the estrogen response element (ERE). On binding to the ER, estrogens exert a wide variety of biological effects, including effects on the development and function of male and female reproductive tissues, bone remodeling, and the cardiovascular system, and have been implicated in breast and uterine cancer. Estrogen-regulated genes therefore represent important therapeutic targets. If expression of estrogen-regulated genes could be effectively suppressed, both the discovery and the elucidation of their roles in various physiological processes would be greatly facilitated. Selective estrogen receptor modulators (SERMs) (reviewed in Ref. 1) and ER mutants displaying a dominant-negative phenotype (2) have been used to suppress ER-induced transcription. However, SERMs can display significant agonist activity in specific tissue or cell backgrounds (3, 4). Recently, a number of hERα mutants displaying a dominant-negative phenotype have been described (2, 5). Although these hERα mutants and SERMs disrupt estrogen-induced transcription, they do not affect basal transcription of estrogen-regulated genes. We therefore designed novel hERα variants for ligand-dependent repression of the transcription of ERE-containing genes.

To create ligand-dependent repressors targeted to ERE-containing genes, we constructed chimeras of ERα and the KRAB (Krüppel-associated box) transcription repression domain (6–9) of the KOX1 protein (also named ZNF10) (7, 8). The KRAB domain is a highly conserved 75-amino acid region found in approximately one-third of the vertebrate Krüppel-like (Cys_2-His_2) zinc finger proteins (6). When tethered to DNA, the KRAB domain suppresses transcription activation mediated by a variety of transcription factors (7, 9–12), represses transcription mediated by all three classes of eukaryotic RNA polymerase (10–12), and functions as a repressor even when bound at DNA sites up to 3 kilobases from the transcription initiation site (10, 11, 13, 14).

In this study, we characterize and examine mechanistically the ability of ER-KRAB domain chimeras to suppress transcription of synthetic genes containing the consensus ERE or the imperfect ERE from the natural pS2 promoter (15). Although the ER-KRAB chimeras were found to exhibit efficient estrogen- or antiestrogen-dependent repression of promoters containing the consensus ERE in several cell and promoter contexts, they were unable to repress transcription from the imperfect ERE found in the pS2 promoter. To achieve repression from a promoter containing the native pS2 ERE, we developed a novel repressor with increased affinity for this imperfect ERE. We recently described the use of a modified p22 challenge phage system to select mutant steroid receptor DNA-binding domains with altered DNA binding specificity and an enhanced affinity for EREs (16). By integrating information obtained from those genetically selected mutant DNA-binding

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[1] The abbreviations used are: ER, estrogen receptor; hER, human estrogen receptor; ERE, estrogen response element; SERMs, selective estrogen receptor modulators; LBD, ligand-binding domain; DBD, DNA-binding domain; CHO, Chinese hamster ovary; OHT, 4-hydroxytamoxifen.

modules with the ligand-regulated ER-KRAB chimeras, we produced a prototype of a new class of targeted gene repressor. This novel ER-KRAB chimera (KERK-3M) is a potent repressor of both basal and estrogen-induced activities of genes containing the consensus ERE or the imperfect pS2 ERE.

EXPERIMENTAL PROCEDURES

Cloning of hERα-KRAB Chimeras—To fuse the Kox1 KRAB domain to hERα, unique *Nhe*I sites were introduced into the hERα cDNA sequence. To facilitate sequence verification after mutagenesis, the following hER fragments from plasmid pCMV5hER were initially subcloned into pGEM11Zf(+) (Promega, Madison, WI): 1) the *Eco*RI/*Not*I N-terminal fragment, 2) the *Not*I/*Hin*dIII fragment containing the LBD, and 3) the *Hin*dIII/*Bam*HI C-terminal fragment of pCMV5hER and pCMV5hERL540Q (3, 17). QuikChange mutagenesis (Stratagene) was then employed to introduce unique *Nhe*I sites into these plasmids, generating the vectors pG11EnsNhe, pG11EnhNhe, pG11EbhNhe, and pG11QbhNhe, respectively. To achieve this, the following primers were used: for pG11EnsNhe, GCCCGCGGCCACGGACCGCTAGCAATGACCATGACCCTCCA (forward) and TGGAGGGTCATGGTCATTGCTAGCGGTCCGTGGCCGCGGGC (reverse); for pG11EnhNhe, AAGTATGGCTATGGAGCTAGCCAAGGAGACTCGCTA (forward) and TAGCGAGTCTCCTTGGCTAGCTCCATAGCCATACTT (reverse); and for pG11EbhNhe and pG11QbhNhe, GAGGCAGAGGGTTTCCTGCTAGCTGCCACAGTCTGAG (forward) and CTCAGACTGTGGCAGCTAGCAGGAAACCCTCTGCCTC (reverse).

The KOX1 cDNA (9) was a kind gift of Dr. Hans-Jürgen Thiesen (University of Rostock, Rostock, Germany). Polymerase chain reaction amplification by *Taq* DNA polymerase (Life Technology, Inc.) generated fragments of the KOX1 (ZNF10) protein (amino acids 1–91) containing both the KRAB A- and B-domains that could be cloned either at the N terminus of hER and ΔA/B-hER (N-KRAB) or at the C terminus of hER and hER L540Q (C-KRAB). The following oligonucleotides were used: N-KRAB, CAGAATTCATGGATGCTAAGTCACTAAC (forward) and TATCTAGAAATGCAGTCTCTGAATCAG (reverse); and C-KRAB, CTTCTAGATATGGATGCTAAGTCACTAAC (forward) and ATGGATCCTAAATGCAGTCTCTGAATCAG (reverse).

The resulting amplified products were subcloned into the pGEM-T vector (Promega). After verifying the sequence, the N-KRAB insert was obtained as an *Eco*RI/*Xba*I fragment and together with the *Nhe*I/*Not*I fragment of plasmid pG11EnsNhe was cloned into pCMV5hER, pCMV5hERL540Q, and pCMV5hERFS digested with *Eco*RI/*Not*I or with the *Nhe*I/*Hin*dIII fragment of pG11EnhNhe into pCMV5hER, pCMV5hERL540Q, and pCMV5hERfs digested with *Eco*RI/*Hin*dIII. These manipulations yielded plasmids pCMV5KER, pCMV5KERQ, pCMV5KERFS, pCMV5K-ΔA/B-ER, pCMV5K-ΔA/B-ERQ, and pCMV5K-ΔA/B-ERFS, respectively. The C-KRAB insert was obtained as an *Xba*I/*Bam*HI fragment and ligated into *Nhe*I/*Bam*HI-digested plasmids pG11EbhNhe and pG11QbhNhe, respectively. The resulting hER LBD-KRAB fusions were then obtained as *Xba*I/*Bam*HI fragments and cloned into similarly digested plasmids pCMV5hER, pCMV5-ΔA/B-hER, pCMV5KER, and pCMV5K-ΔA/B-ER. These manipulations yielded plasmids pCMV5ERK, pCMV5ERQK, pCMV5-ΔA/B-ERK, pCMV5-ΔA/B-ERQK, pCMV5KERK, pCMV5KERQK, pCMV5K-ΔA/B-ERK, and pCMV5K-ΔA/B-ERQK, respectively.

To establish that ERE binding is required for transcription repression by the ER-KRAB chimera, its wild-type hER DNA-binding domain was replaced through exchange of the respective *Not*I/*Hin*dIII fragments with a mutated DNA-binding domain. This latter DBD no longer recognizes the ERE sequence due to the E203G, G204S, and A207V mutations in the DNA recognition helix (5, 17). To establish that a functional KRAB domain is required for transcription repression, the previously reported E26A, E27A, and E28A mutations (7) were introduced into the KRAB domain of the ER-KRAB chimera with the QuikChange protocol using the following oligonucleotides: GACTTCACCAGGGCGGCCGCGAAGCTGCTGGAC (forward) and GTCCAGCAGCTTCGCGGCCGCCCTGGTGAAGTC (reverse).

A FLAG-GAL4-KRAB chimera was constructed to serve as a control. Dr. C. M. Chiang (University of Illinois) provided us with a FLAG-GAL4-VP16 fusion construct in the bacterial expression plasmid pET11d (Novagen). We obtained the FLAG-GAL4-VP16 coding sequence by digestion with *Nco*I and subsequent fill in with *Pfu* polymerase followed by *Bam*HI digestion to liberate the insert. The gel-purified fragment was then ligated into the mammalian expression vector pcDNA3 (Stratagene) to generate plasmid pFGVP16. For this purpose, pcDNA3 was initially digested with *Hin*dIII, filled in with *Pfu* polymerase, and subsequently digested with *Bam*HI. The GAL4 C terminus was obtained in conjunction with a polylinker as a polymerase chain reaction fragment from plasmid pM (CLONTECH), changing the Dam methylation-sensitive *Bcl*I site into an *Apa*I site in the process. The polymerase chain reaction fragment was digested with *Xho*I/*Apa*I and ligated into similarly digested plasmid pFGVP16 to generate plasmid pFGmcs. In our transfections, this plasmid is referred to as GAL4. The above described N-terminal KRAB domain, obtained as an *Eco*RI/*Bam*HI fragment, was ligated into plasmid pFGmcs, which provided the stop codon, generating the vector pFGK.

Plasmid $(ERE)_4$-pGL3-SV40PE that we constructed served as an indicator of repression. This plasmid is derived from plasmid pGL3-Control (Promega) and contains four consensus EREs upstream of the SV40 promoter, which renders the plasmid estrogen-responsive. The SV40 promoter and enhancer in this plasmid constitutively drive the expression of firefly luciferase; therefore, both activation and repression can be studied effectively. The estrogen response elements were obtained from plasmid $(ERE)_4$-TATA-CAT (18), which was digested with *Hin*dIII, blunt-ended with *Pfu* polymerase, and religated to generate an *Nhe*I site. An *Nhe*I/*Bgl*II digest was then performed to liberate the EREs. This fragment was ligated into similarly digested vector pGL3-Control. Another series of pGL3-Control-based reporters was constructed containing one, two, and four EREs, respectively. To achieve this, an extraneous *Bgl*II site was removed from the multiple cloning site of plasmids pGL3-$(ERE)_1$-TATA, pGL3-$(ERE)_2$-TATA, and pGL3-$(ERE)_4$-TATA (16) by *Hin*dIII/*Xho*I digestion and subsequent religation after *Pfu* DNA polymerase-mediated fill in. Following this treatment, the *Bgl*II/*Sal*I backbone fragment containing the respective number of EREs was ligated to the *Bgl*II/*Sal*I fragment of *Bgl*II/*Pvu*I/*Sal*I-digested plasmid pGL3-Control. To test the ER-KRAB chimeras in a non-SV40-based promoter/enhancer context, plasmids $(ERE)_4$-PGL3-TK and $(ERE)_4$-PGL3-EF1α were constructed. Plasmid pGL3-TK was constructed by inserting the thymidine kinase promoter/enhancer as a *Bgl*II/*Hin*dIII fragment obtained from plasmid pRL-TK (Promega) into similarly digested plasmid pGL3-Basic (Promega). Plasmid pGL3-EF-1α was constructed by inserting the elongation factor 1α promoter/enhancer obtained as a *Hin*dIII/*Nco*I fragment from plasmid pEFmyc/nuc (Invitrogen) into similarly digested plasmid pGL3-Basic. These plasmids were then made estrogen-responsive by incorporating four copies of the ERE obtained as an *Nhe*I/*Bgl*II fragment from plasmid pGL3-$(ERE)_4$-TATA. To test the ability of ER-KRAB chimeras to repress transcription from a single non-consensus ERE, a 345-base pair *Sac*I/*Sma*I fragment containing the pS2 ERE was isolated from the pS2 promoter and inserted into similarly digested plasmid pGL3-Promoter, resulting in plasmid pGL3-pS2-SV40P. Plasmid pGL3-(pS2 $ERE)_1$-TATA is derived from the pGL3-$(ERE)_1$-TATA reporter by mutation of 2 base pairs in the consensus ERE. The imperfect ERE created, 5′-AGGTCActgTGGCCC-3′, is the ERE in the pS2 5′-flanking region. For studies with the FLAG-GAL4-KRAB fusions, the repression reporter plasmid G_5-pGL3-Control was constructed by inserting five GAL4-binding sites obtained as an *Xho*I/*Bam*HI fragment from plasmid pG5E1b (19) into *Xho*I/*Bgl*II-digested plasmid pGL3-Control.

Cell Maintenance, Transfection, and Reporter Gene Assays—HepG2 human hepatoma cells and HeLa cells were maintained in a humidified 5% CO_2-containing environment at 37 °C in Dulbecco's minimal essential medium (Sigma) supplemented with 10% charcoal/dextran-stripped fetal bovine serum (Atlanta Biologicals, Inc., Atlanta, GA) 50,000 units/liter penicillin, and 50 mg/liter streptomycin (Life Technologies, Inc.). Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (1:1; Sigma), 29.2 mg/liter L-glutamine (Sigma), 5% charcoal/dextran-stripped newborn bovine serum (Atlanta Biologicals, Inc.), 50,000 IU/liter penicillin, and 50 mg/liter streptomycin. MCF-7 cells were maintained in Eagle's minimal essential medium plus phenol red supplemented with 5% newborn calf serum, 50,000 IU/liter penicillin, and 50 mg/liter streptomycin. At least 2 days prior to the experiment, cells were transferred to 1:1 Dulbecco's modified Eagle's medium/nutrient mixture F-12, 29.2 mg/liter L-glutamine, 5% charcoal/dextran-stripped newborn bovine serum, 50,000 IU/liter penicillin, and 50 mg/liter streptomycin.

Transient transfections were carried out by the calcium phosphate coprecipitation method. Briefly, cells were plated in 60-mm dishes at a density of 4.5×10^5 cells/dish for HepG2 cells and 2.5×10^5 cells/dish for CHO cells, in 6-well plates at 1.0×10^5 cells/well, or in 12-well plates at 5×10^4 cells/well. The next day, the medium was replaced; and 2–6 h later, calcium phosphate crystals were added. 12–16 h later, the cells were subjected to a 3-min shock with 20% glycerol in Tris-buffered saline, pH 7.4. The medium was replaced; and where appropriate, hormone was added to the indicated concentrations. The cells were harvested 48 h later for the reporter gene assay by addition of appro-

priate amounts of passive lysis buffer (Promega). The activity of the resulting extracts was determined using the dual luciferase assay protocol (Promega) according to the manufacturer's directions on a Monolight 2010 luminometer.

RESULTS

hER-KRAB-mediated Repression Requires Ligand, EREs, and a Functional KRAB Domain—To produce the KRAB-hERα-KRAB (KERK) construct (see Fig. 2*B*), the complete KRAB repressor domain (containing both the KRAB A- and B-domains) was placed at both the N and C termini of hERα. The ability of KERK to repress transcription of a reporter gene containing the SV40 promoter and enhancer (SV40PE) and four consensus EREs was tested. This $(ERE)_4$-pGL3-SV40PE reporter plasmid exhibits substantial intrinsic activity, referred to as basal transcription, which is further enhanced by ligand-activated ER. To establish the effect of ligand on the ability of a KRAB construct to repress transcription, transient transfections were carried out in ER-negative HepG2 human hepatoma cells in the presence or absence of the estrogen moxestrol, which liver cells metabolize more slowly than 17β-estradiol (20). The basal promoter activity of the $(ERE)_4$-pGL3-SV40PE reporter plasmid in the absence of estrogen receptor was set at 100%. Cotransfected hERα expression plasmid elicited a moxestrol-dependent 3–4-fold induction of luciferase activity (Fig. 1*A*), whereas increasing amounts of unliganded ER did not affect transcription. In the absence of an ER ligand and at 20 ng of transfected KERK expression plasmid, there was a modest 1.6-fold repression of transcription. However, full repression (4.8-fold) required the presence of ligand (Fig. 1*A*). Since KRAB repression was largely ligand-dependent, subsequent studies were carried out in the presence of ligand.

The sequence specificity of repression was shown by the inability of an hERα-KRAB (ERK) chimera (shown in Fig. 2, *A* and *B*) to repress transcription from the five GAL4-binding sites in the G_5-pGL3-SV40PE reporter (Fig. 1*B*) and by the inability of GAL4-KRAB to repress transcription from the four EREs in the $(ERE)_4$-pGL3-SV40PE reporter (Fig. 1*C*). The reporters were functional since GAL4-KRAB repressed transcription by >90% from the G_5-pGL3-SV40PE reporter (Fig. 1*B*), whereas hER activated basal transcription by 3.8-fold and ERK repressed transcription by 4.5-fold on the $(ERE)_4$-pGL3-SV40PE reporter (Fig. 1*C*). The issue of DNA binding specificity was also addressed by introducing mutations into the DNA recognition helix of the hER DBD that shift the specificity from the ERE to the glucocorticoid response element and thereby prevent binding to the ERE (5, 17). This chimera (ERKmutDBD) no longer repressed transcription on either of the reporter plasmids (Fig. 1, *B* and *C*). As expected, introducing the mutations E26A, E27A, and E28A into the KRAB domain (7) of ERK (ERKmutKRAB) abolished repression (Fig. 1*C*).

Influence of Ligand and Estrogen Receptor AF1 and AF2 Mutations on KRAB Repression—Although the mechanism of transcription repression by the KRAB domain is not fully understood, KRAB has been shown to interact with the human corepressors TIF1α and TIF1β (also isolated as KAP-1) and their murine homologue KRIP-1 (13, 21, 22). Interestingly, TIF1α (23), but not TIF1β, is thought to act as a coactivator of steroid receptor-mediated transcription activation by interacting with the AF2 region of ligand-occupied steroid receptors. The interactions of TIF1α with the KRAB domain and with the AF2 region of steroid receptors take place via two distinct interaction domains found within the TIF1α protein and might interfere with the ability of the KRAB domain to function as a repressor in the presence of AF2. It was therefore of interest to examine whether presenting the KRAB domain in different ways in the context of estrogen receptor chimeras would favor a functional interaction of KRAB and its corepressors, thereby enabling the KRAB domain to operate more effectively as a transcription repressor.

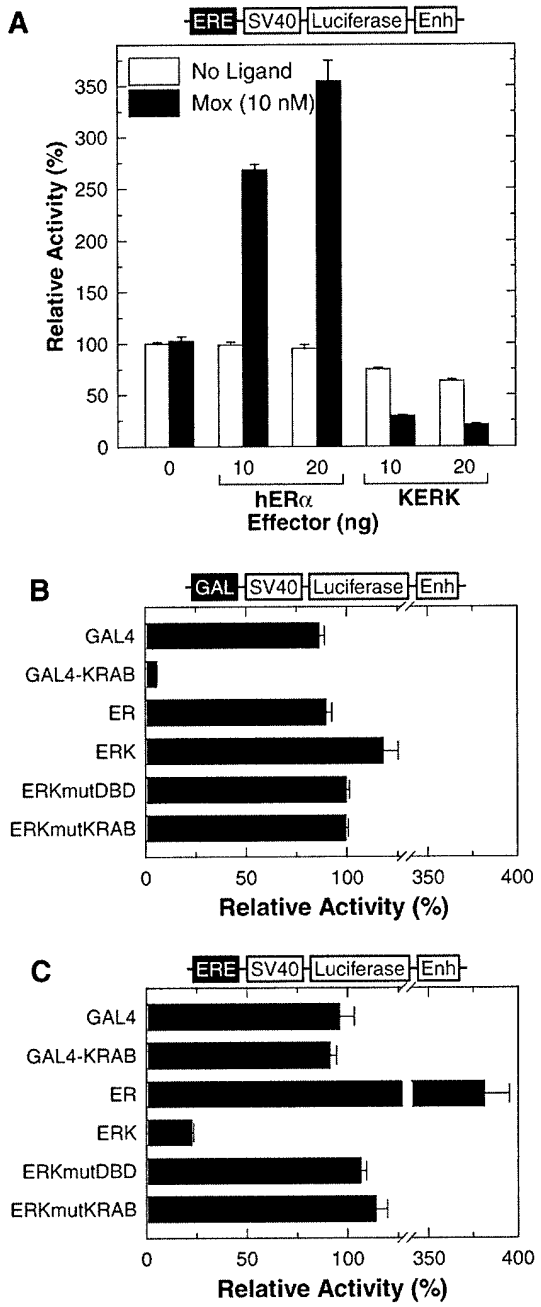

FIG. 1. **Repression by ERK is DNA sequence-specific and requires ligand and a functional KRAB domain.** *A*, transcription repression properties of the KERK chimera and activation properties of hERα on the $(ERE)_4$-pGL3-SV40PE reporter plasmid in HepG2 cells in the absence and presence of 10 nM moxestrol (*Mox*). All experiments were carried out in the presence of 10 nM moxestrol, except where noted. Luciferase activity from the transfected reporter was determined as described under "Experimental Procedures." The activity of the reporter plasmid alone was normalized to 100 kilo-luciferase units. To establish whether both sequence-specific DNA binding and a functional KRAB domain are required for repression by the ERK chimera, the effects on transcription from the $(G)_5$-pGL3-SV40PE and $(ERE)_4$-pGL3-SV40PE reporter plasmids in HepG2 cells were examined by cotransfection of the indicated GAL4 DBD- and hER-based effector constructs (*B* and *C*, respectively). The data obtained were normalized against the luciferase activity of the indicated reporter plasmid alone. The data in *A–C* represent the mean ± S.E. of at least three independent transfections. *Enh*, enhancer.

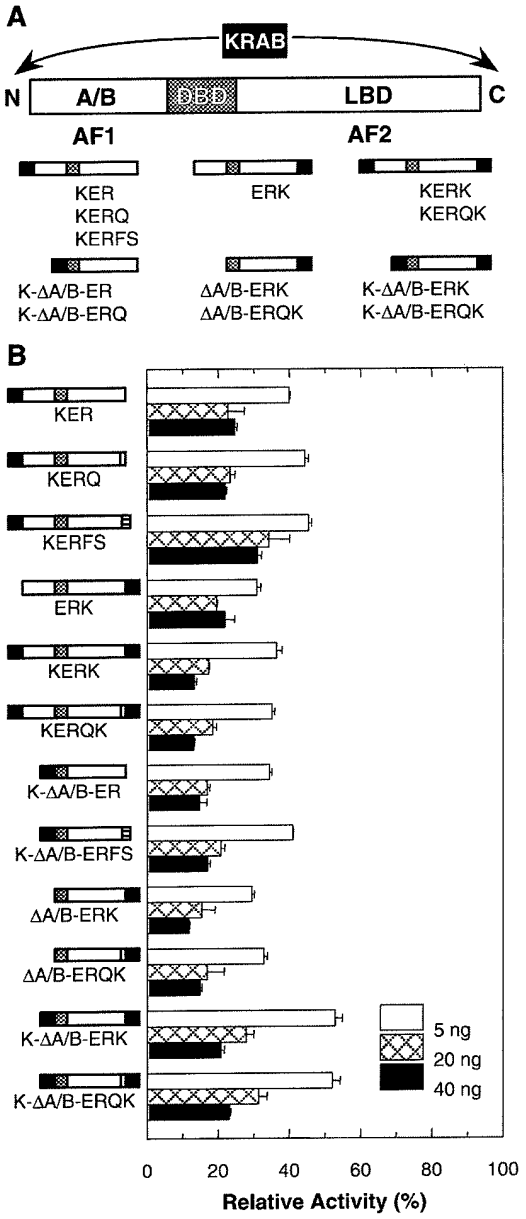

FIG. 2. **Influence of AF1 and AF2 on repression properties of hER-KRAB chimeras.** *A*, the KRAB domain was fused in frame at either the N or C terminus and at both termini of full-length wild-type hERα, at the N terminus of several hERα mutants in which the ligand-independent activation function (AF1) was removed through deletion of the A/B-domain (ΔA/B) or in which the ligand-dependent activation function (AF2) was ablated by point mutations L540Q (*Q*) and S554fs (*FS*), or a combination of these two classes of mutations. In the constructs, the DBD is indicated as a *shaded box*, and the AF2 mutations in the LBD are indicated as *Q* (L540Q) and *FS* (S554fs), respectively. Ablation of AF1 activity, achieved through deletion of the first 178 amino acids of hERα, is indicated as ΔA/B. The KRAB repressor domain is indicated as a *black box*. *B*, increasing amounts (5, 20, or 40 ng) of the expression plasmids encoding the hER-KRAB chimeras were transfected into HepG2 cells using the $(ERE)_4$-pGL3-SV40PE plasmid as a reporter. A *vertical line* in the ER LBD indicates the L540Q point mutation (*Q*), whereas the *striped box* extending the LBD C terminus represents the additional amino acid sequence introduced by the S554fs frameshift mutation (*FS*). The data obtained were normalized against the luciferase activity of the reporter plasmid alone, which was set at 100%. The data in *B* represent the mean ± S.E. of at least three independent transfections.

To analyze the effect of position and the influence of the ER activation domains on KRAB repression, the KRAB domain was fused in frame at either the N or C terminus and at both ends of hERα (Fig. 2, *A* and *B*). To prevent interaction with steroid receptor coactivators, we also employed a number of hERα mutants in which AF1 and/or AF2 activity was ablated. Since the ligand-independent activation function AF1 is spread through much of the A/B-domain of hERα (24, 25), AF1 ablation was achieved by deleting the entire A/B-domain (amino acids 1–178, indicated as ΔA/B). Removal of the ligand-dependent activation function AF2 was achieved through introduction of either of two point mutations in the ligand-binding domain, L540Q and S554fs (Q and FS, respectively) (Fig. 2*A*). These mutations confer a dominant-negative phenotype on hERα (2), which might further potentiate transcription repression by the KRAB domain.

The ability of the ER-KRAB chimeras to repress transcription was determined by cotransfecting the $(ERE)_4$-pGL3-SV40PE reporter plasmid and increasing amounts (5, 20, or 40 ng) of the expression plasmid encoding each KRAB chimera into HepG2 cells in the presence of 10 nM moxestrol (Fig. 2*B*). Even at the lowest amount transfected, all of the chimeras achieved at least 45% repression, and most achieved >55% repression. The differences in repression among the various constructs were modest. All of the ER-KRAB chimeras are therefore effective transcription repressors. Surprisingly, ablation of AF1 and/or AF2 activity had little or no effect on the extent of KRAB repression. For example, at 40 ng of transfected expression plasmid, the AF2-containing chimera KER repressed transcription by 75%. Ablation of AF2 by the L540Q mutation in the KERQ chimera or by the S554fs mutation in the KERFS chimera (2) had little effect on the magnitude of transcription repression. Deletion of AF1 modestly enhanced repression only when the KRAB domain was present at the C terminus of the protein. At 40 ng of transfected expression plasmid, the ERK and ΔA/B-ERK constructs repressed transcription by 78 and 88%, respectively. The KERK, KERQK and ΔA/B-ERK constructs were the most effective, with each repressing transcription by 87–88%. Since these differences were negligible, we elected to use the KERK repressor in subsequent experiments.

KRAB-mediated Repression Is Not Blocked by Trichostatin A—It has been proposed that KRAB repression is mediated through recruitment of the corepressors TIF1α and TIF1β. These proteins contain RBCC (RING finger-B boxes-coiled coil), PHD finger, and bromodomain interaction domains. Since these domains are also found in complexes implicated in chromatin-mediated transcription repression, it has been suggested that KRAB may act by modifying chromatin to achieve a repressive state (21, 26). Many chromatin modifiers recruit histone deacetylases or contain intrinsic histone deacetylase activity. The histone deacetylase inhibitor trichostatin A has been widely used to identify chromatin events based on histone deacetylation (27, 28). Addition of 0.25 or 1 μM trichostatin A had no effect on the ability of the KERK or GAL4-KRAB chimeras to repress transcription from several reporter genes (Fig. 3). Although trichostatin A failed to affect KRAB repression, it is functional in HepG2 cells, as judged by its ability to strongly potentiate moxestrol/ER-mediated transcription of a stably integrated vitellogenin promoter in HepG2 cells.[2]

Effect of Cell Line, Promoter, and Ligand on ER-KRAB Repression—We wanted to determine whether KRAB repression was equally effective in different cell lines on strong and weak promoters and whether the KRAB chimera could repress transcription in the presence of wild-type ERα or ERβ (29, 30). To examine the effect of promoter strength on KRAB repression, repression was evaluated in reporter genes containing the rel-

[2] C. Mao and D. J. Shapiro, submitted for publication.

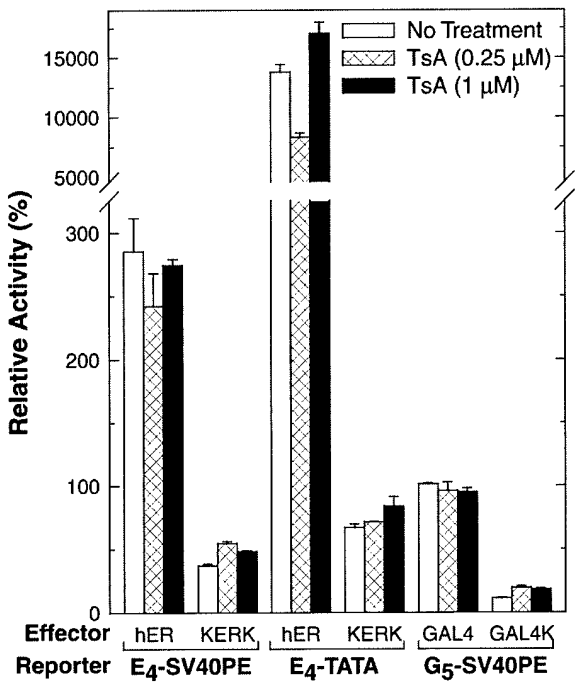

FIG. 3. **Trichostatin A does not influence repression by KRAB chimeras.** To establish whether trichostatin A (*TsA*) could relieve KRAB-mediated repression, we cotransfected reporter plasmids $(ERE)_4$-pGL3-SV40PE and G_5-pGL3-SV40PE and the indicated hER- and GAL4 DBD-based effector constructs, respectively, in the absence (*open bars*) or presence (0.25 μM, *cross-hatched bars*; 1 μM, *filled bars*) of trichostatin A. Moxestrol (10 nM) was present when hER or KERK was used. Where appropriate, trichostatin was added 24 h prior to harvest of the HepG2 cells. The data represent the mean ± S.E. of at least three independent transfections.

atively weak thymidine kinase promoter, the strong SV40 promoter/enhancer (SV40PE), and the extremely powerful elongation factor 1α promoter. Repression in the presence of endogenous ER was determined by cotransfecting plasmids encoding wild-type ERα or ERβ into the cells along with the KERK expression plasmid. Even though we used three times more hERβ expression plasmid than hERα expression plasmid, in agreement with earlier studies (29, 31), hERβ was significantly less effective in activating transcription than hERα (Fig. 4, *A* and *D*; 3.3-fold *versus* 15-fold in Fig. 4*A*; note that the *ordinate* of *A* is set on a logarithmic scale).

There was an inverse correlation between promoter strength and the additional contribution to promoter activity due to hERα-activated transcription. hERα increased transcription 15-, 2.6-, and 0.9-fold on the $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40, and $(ERE)_4$-pGL3-EF-1α reporter plasmids, respectively. However, on all promoters, in both HepG2 cells (Fig. 4, *A–C*) and CHO cells (Fig. 4, *D–F*), increasing amounts of transfected KERK repressed all, or nearly all, of the hERα- or hERβ-induced activity and most of the basal promoter activity. In the absence of hER, KERK repressed up to 82–92% of basal promoter activity on these reporter plasmids. When transfected at a 3-fold excess relative to hERα, KERK repressed thymidine kinase promoter activity to 45% of the basal thymidine kinase promoter activity, which is a 33-fold reduction from the hERα-induced level of transcription (Fig. 4*A*).

In CHO cells, we tested the thymidine kinase, SV40, and *Xenopus* vitellogenin B1 promoters using the $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40PE, and pGL3-EREVIT reporter plasmids, respectively. These experiments suggested an interesting difference between transcription activation and repression. The EREVIT promoter contained only one consensus ERE, two functional imperfect EREs, and one nonfunctional imperfect ERE (32). The other test promoters contained four consensus EREs. In CHO cells, hERα activated transcription more powerfully from the EREVIT promoter than from the other test promoters (3.4-fold *versus* 1.7–1.9-fold). However, transcription repression by the KRAB chimera was more closely correlated with the number of consensus EREs, and repression was somewhat more effective with the $(ERE)_4$-pGL3-TK and $(ERE)_4$-pGL3-SV40PE reporters than with the pGL3-EREVIT reporter. At a 1:1 ratio of transfected KERK and hERα, repression was clearly dominant, as activity was reduced 3.3–3.6-fold relative to the activity in the presence of hERα alone (Fig. 4, *D–F*). Similar results were obtained when repression by KERK from the $(ERE)_4$-pGL3-TK and $(ERE)_4$-pGL3-SV40PE reporter genes was evaluated in the ER-negative breast cancer cell line MDA-MB231 and in HeLa cells (data not shown).

To evaluate the ability of a KRAB chimera to repress transcription in cells containing high levels of endogenous ER, we tested the effectiveness of the KERK chimera in ER-positive MCF-7 human breast cancer cells (Fig. 5). The ability of SERMs to act as KERK ligands to potentiate KRAB repression was also tested. SERMs, which are mixed agonists/antagonists such as 4-hydroxytamoxifen (OHT), prevent the ER ligand-binding domain from adopting the conformation required for interaction with AF2-dependent coactivators (33), but do not interfere with DNA binding. "Pure" antiestrogens such as ICI 182,780 and RU 58,668 are thought to alter cytoplasmic-nuclear shuttling of hERα and to increase receptor degradation (34–36) and might be expected to impair the ability of ER-KRAB chimeras to repress transcription. To facilitate comparisons of the ability of the different ligands to induce repression, we set luciferase activity in the absence of transfected KERK equal to 100% for each ligand. Repression was not affected by the type of ligand used. Transcription was repressed by 75–89% in the presence of 17β-estradiol, OHT, or ICI 182,780. Surprisingly, repression was most effective when ICI 182,780 was present, indicating that KERK·ICI 182,780 complexes are not rapidly degraded and translocate into the nucleus and bind to ERE-containing DNA. OHT and ICI 182,780 also elicited efficient repression as KERK ligands in the estrogen receptor-negative HepG2 cell line (data not shown), indicating that repression was not due to the SERMs interfering with hER-mediated transcription activation.

Effect of the Number of EREs and ERE Binding Affinity on Transcription Repression—Virtually all studies employing KRAB repressors have utilized conditions favorable to repression in which the KRAB chimera binds to synthetic constructs containing multiple copies of a perfect DNA-binding site (7–12, 14, 37, 38). Since KERK repressed expression from the ER-EVIT promoter (which contains a single consensus ERE and three additional non-consensus EREs) less effectively than it repressed promoters containing four consensus EREs (Fig. 4, *D–F*), it was of interest to establish the minimum number of consensus EREs required for repression. We therefore constructed SV40-based reporter genes containing one, two, and four EREs and examined the ability of transfected KERK to repress their transcription (Fig. 6*A*). Repression was similar for the reporter genes containing two or four EREs and reached a plateau at 87%. Although repression from the reporter gene containing a single ERE was dose-dependent, the inability to reduce promoter activity below ~30% of basal activity, even at high levels of transfected KERK, was troubling (Fig. 6*B*). We therefore set out to enhance the potential of KERK to repress transcription.

Through the use of a modified form of the bacteriophage p22 challenge phage selection system (39), our laboratory recently identified progesterone receptor DNA-binding domain muta-

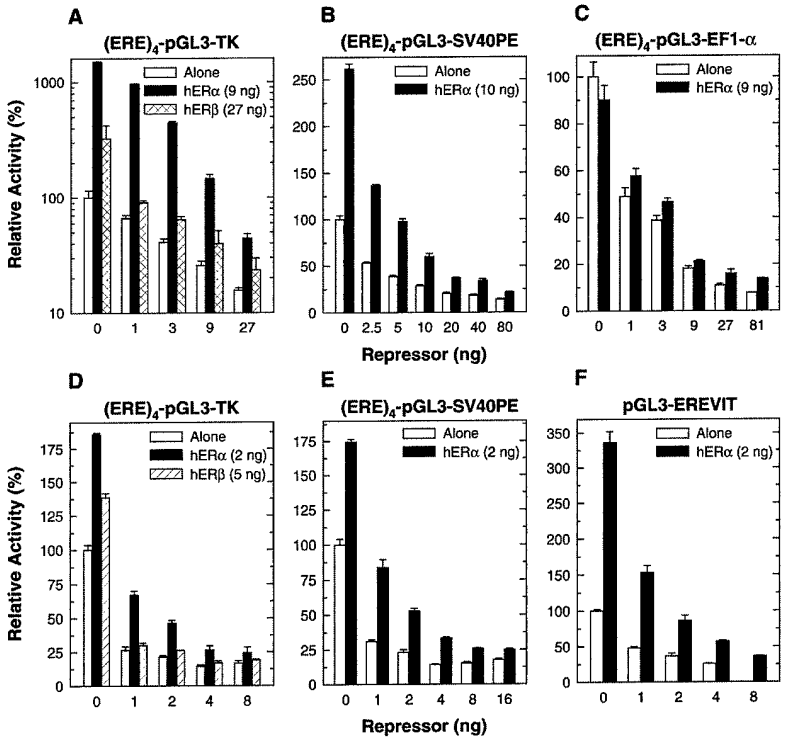

FIG. 4. **KERK effectively represses transcription on several estrogen-responsive promoters in HepG2 and CHO cells in the presence and absence of hER.** Repression was assessed in the presence of 10 nM moxestrol in the presence and absence of the indicated amounts of cotransfected hERα or hERβ expression plasmids using reporter plasmids $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40PE, and $(ERE)_4$-pGL3-EF-1α in HepG2 cells (*A–C*, respectively) and plasmids $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40PE, and pGL3-EREVIT in CHO cells (*D–F*, respectively). The transfections and luciferase assays were carried out as described under "Experimental Procedures." The data represent the mean ± S.E. of at least three independent transfections normalized to the activity of the indicated reporter plasmid alone, which was set equal to 100%.

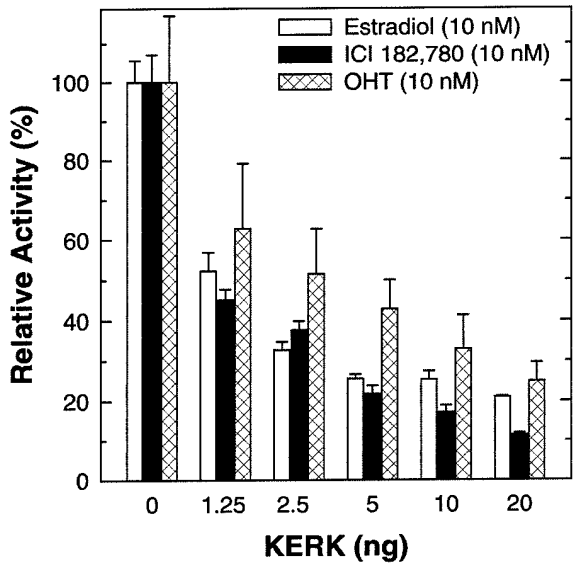

FIG. 5. **Antiestrogens induce repression by KERK in MCF-7 human breast cancer cells.** Repression was assessed on the $(ERE)_4$-pGL3-SV40PE reporter plasmid in the presence of 17β-estradiol (10 nM), OHT (10 nM), or ICI 182,780 (10 nM). Transfections and luciferase assays were carried out as described under "Experimental Procedures." Since the different effects of agonists and antagonists on the growth of MCF-7 cells influenced the activity of the internal standard, to facilitate comparisons, the data obtained for each individual treatment group were normalized against the luciferase activity of the reporter plasmid alone in the absence of transfected chimera, which was set at 100%. The average luciferase units for each treatment were as follows: no ligand, 170,000; 17β-estradiol, 39,000; ICI 182,780, 60,000; and OHT, 198,000. The data represent the mean ± S.E. of at least three independent transfections.

tions that changed the DNA binding specificity from the glucocorticoid response element/progesterone receptor element to the ERE and that resulted in enhanced binding to the consensus ERE and to the imperfect ERE in the pS2 gene (16). One of the progesterone receptor DBD mutants we isolated, DBD5, exhibited >10-fold higher affinity than the wild-type ER DBD for the consensus and pS2 EREs. We reasoned that enhancing the ability of KERK to bind to the ERE might potentiate its transcription repression properties. Therefore, the corresponding three mutations (E203W, Q214A, and H216G) from the progesterone receptor DBD5 mutant were introduced into the DNA-binding domain of KERK, resulting in KERK-3M. We compared the ability of KERK-3M and KERK to repress transcription from the promoter containing a single ERE. KERK-3M was a more potent repressor than KERK. Almost 2-fold less transfected KERK-3M was required to reach a given level of repression, and the extent of repression by KERK-3M increased progressively at all of the amounts tested (Fig. 6*B*).

KERK-3M, but Not KERK, Effectively Represses Transcription from a Promoter Containing the Imperfect pS2 ERE—Although the above studies demonstrate that KERK and KERK-3M are able to repress transcription from a single consensus ERE, most estrogen-regulated genes contain imperfect EREs. To test repression from an ERE in a native gene, we elected to use a fragment from the estrogen-inducible pS2 gene that contains the single imperfect ERE (5′-AGGTCActgTG-GCCC-3′) responsible for the strong estrogen induction of pS2 gene expression. Although pS2 is a clinical and prognostic marker for hormone-responsive breast cancer (40), the function of pS2 and its role in breast cancer development and progression remain poorly understood.

In vitro DNA binding and *in vivo* transactivation by wild-type ER and by the ER DBD are both substantially reduced when the non-consensus pS2 ERE is present rather than the consensus ERE (41). Since binding of the ER to an imperfect ERE is difficult to study directly in intact cells, as a test of pS2 ERE-ER interaction, we tested ER-mediated transactivation from a single pS2 ERE. We inserted the three up-binding mutations used in the KERK-3M repressor (E203W, Q214A, and H216G) into the DBD of wild-type hERα (hER-3M) and assessed the ability of the resulting hER-3M to activate transcription from the pS2 ERE. Relative to wild-type hER, 10 or 50 ng of transfected hER-3M increased transactivation from the pS2 promoter by 2.5- and 1.9-fold, respectively ($n = 6$; data not shown). This supports the view that these mutations enhance

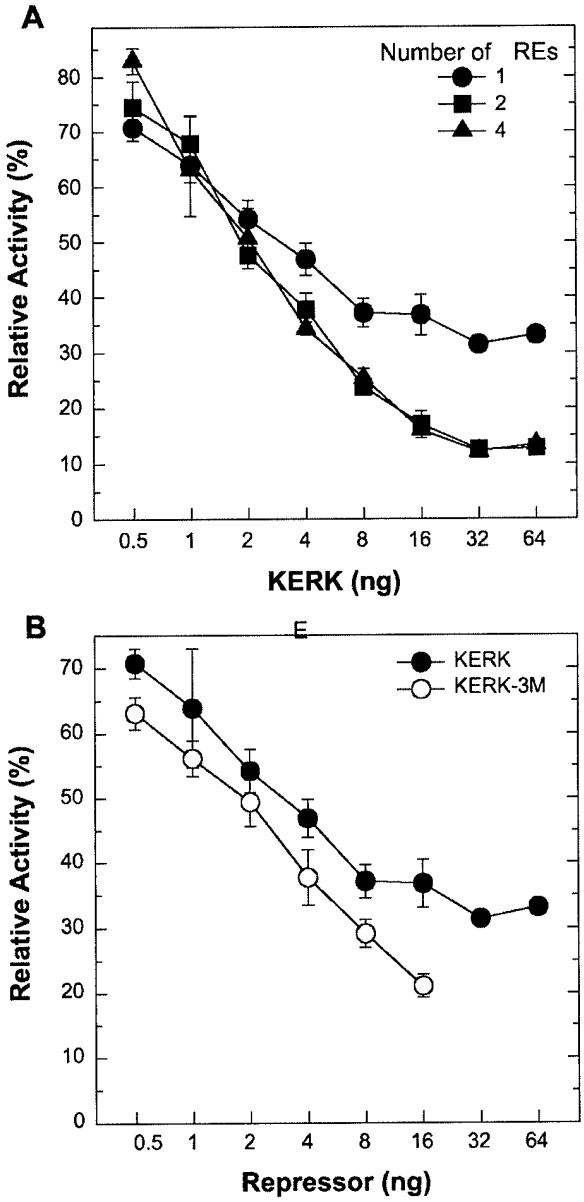

FIG. 6. **Repression from a single consensus ERE is increased when ERE binding by the chimera is enhanced.** Transfections and luciferase assays were carried out as described under "Experimental Procedures." In all cases, the data obtained were normalized against the luciferase activity of the indicated reporter plasmid alone, which was set at 100%. *A*, transcription repression by the KERK chimera was assessed in HepG2 cells on pGL3-SV40PE reporter plasmids containing the indicated number of EREs (*REs*). *B*, repression by KERK and by a mutant KERK possessing increased DNA binding (KERK-3M) was assessed in HepG2 cells on the $(ERE)_1$-pGL3-SV40PE plasmid. Note that the data for the KERK chimera are also shown in *A*. Because of its enhanced effectiveness as a repressor, 16 ng was the highest level of KERK-3M tested. The data represent the mean ± S.E. of at least three independent transfections.

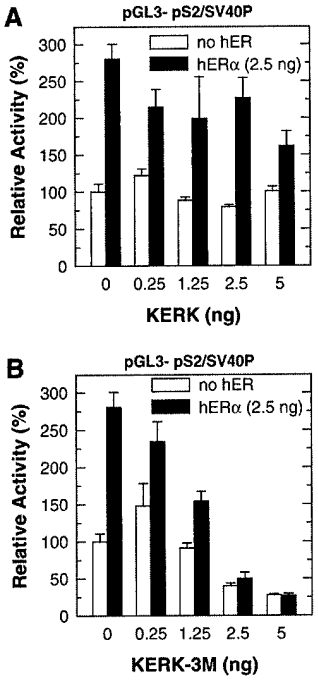

FIG. 7. **Transcription repression from a promoter containing the pS2 ERE.** The pGL3-pS2-SV40P reporter gene was transfected into HepG2 cells in the presence or absence of cotransfected hER and 10 nM moxestrol. The activity of the reporter gene in the absence of any transfected repressor or hER was set at 100%. *A*, repression by the indicated amounts of transfected KERK expression plasmid; *B*, repression by the KERK-3M plasmid. Since this experiment was carried out with cells plated in smaller wells than in the study in Fig. 6, 1 ng of transfected KRAB chimera expression plasmid in this study corresponds to ~2.5 ng of transfected DNA in the study shown in Fig. 6. The data represent the mean ± S.E. of at least three independent transfections.

in vivo binding of the ER to the pS2 ERE.

To evaluate whether KERK and KERK-3M could repress transcription from an imperfect ERE in a native promoter context, we constructed a pS2-based reporter gene using the 345-nucleotide fragment from the pS2 promoter that contains the pS2 ERE (15). KERK only weakly repressed moxestrol/hER-induced transcription of the pS2-based reporter and was unable to repress basal transcription of the reporter (Fig. 7*A*). In striking contrast, the KERK-3M chimera effectively repressed all of the moxestrol/hER-induced transcription and elicited a strong dose-dependent repression of basal promoter activity (Fig. 7*B*). These data indicate that use of a genetically selected set of up-binding mutations strongly potentiates the ability of ER-KRAB chimeras to repress transcription from a naturally occurring imperfect ERE.

DISCUSSION

ER-KRAB Chimeras Containing ER Activation Domains Repress Transcription—In a study of repression of the human immunodeficiency virus type 1 long terminal repeat, dominant-negative Tat mutants linked to KRAB were far more effective repressors than Tat-KRAB chimeras retaining an active Tat transactivation domain (42). In a similar way, ER activation domains could interfere with KRAB repressor activity since the putative KRAB corepressor TIF1α acts as a coactivator on interaction with the AF2 domain of ligand-occupied ER (23). Deleting or mutating one or both ER transactivation domains did not enhance repression of transcription, indicating that the KRAB domain is dominant over the ER transactivation domains and can overcome the activity of any ER coactivators still able to bind the ER-KRAB chimeras.

KERK Represses Transcription when Wild-type ER Is Present—If the ER-KRAB chimeras and wild-type ER have similar affinities for the ERE, it seemed plausible that wild-type ER could compete effectively for binding to the EREs in our reporter genes and might block the ability of the KRAB chimeras to repress transcription. Consistent with our finding that the KRAB domain is dominant over the AF1 and AF2 domains, we found that KERK effectively represses transcription in the presence of either hERα or hERβ in several cell and promoter contexts (Fig. 4).

Not only can KERK repress transcription in the presence of ER, it also represses transcription of the powerful $(ERE)_4$-pGL3-EF-1α reporter, whose expression is not up-regulated by the ER. In the progression of breast cancers to an estrogen-independent phenotype in which antiestrogens no longer limit their growth, it has been suggested that genes that were initially estrogen-regulated become constitutively active (43–45). The $(ERE)_4$-pGL3-EF-1α construct serves as a prototype for this class of genes. KERK effectively suppresses the high level of basal transcription from this promoter (Fig. 4*C*).

An ER Ligand Is Required for Repression—The role of ligand in ERE binding by the ER has been controversial (reviewed in Ref. 46). Although most studies support the view that liganded ER binds with higher affinity to the ERE than unliganded ER, variable levels of ERE binding by unliganded ER have been reported using promoter interference assays (46–48). We observed a minimum level of repression with unliganded KERK (Fig. 1*A*). The presence of ER ligands that are either agonists or antagonists strongly potentiated repression by KERK. Since ER-KRAB chimeras in which the KRAB domain was linked to either the N or C terminus had equal potency (Fig. 2*B*), the presence of the large KRAB repressor domain linked to the C terminus of the ER does not appear to limit the access of ligand to the binding pocket.

The mechanisms by which pure antiestrogens such as ICI 182,780 interfere with ER-mediated transcription have been the subject of considerable interest (49). The ER occupied by pure antiestrogens is thought to be largely localized in the cytoplasm (34, 35, 50), where it is rapidly destroyed (34, 35), depleting cellular ER. Although ICI 182,780-occupied receptor binds DNA *in vitro* with slowed kinetics (51), *in vivo*, at least part of the receptor population retains the ability to bind to the ERE (48). Since KERK displayed a similar dose-dependent repression curve when liganded by 17β-estradiol, OHT, or ICI 182,780, our data suggest that even ICI 182,780 induces KERK binding to the ERE. The putative KRAB corepressor TIF1α may potentiate nuclear localization of ICI 182,780-occupied KERK. In a study using an ER mutant missing the nuclear localization signal, the ER coactivator TIF1α allowed ligand-dependent nuclear localization (23).

The Histone Deacetylase Inhibitor Trichostatin A Does Not Interfere with Repression by the KRAB Domain—One possible explanation for the ability of the KRAB domain to repress transcription is that it recruits a corepressor complex containing histone deacetylase activity. Since the histone deacetylase inhibitor trichostatin A (27, 28) had not previously been used in conjunction with the KRAB repressor, we examined its ability to interfere with repression by the KRAB domain. Trichostatin A did not affect repression by two KRAB chimeras on several promoters. Under these conditions, which employ transient transfections, KRAB repression uses a pathway independent of histone deacetylation. One possible explanation for these data is that the maintenance of a repressed chromatin state by the KRAB domain involves the heterochromatin-enriched factors HP1a, MOD1, and MOD2, which reportedly interact with KRAB corepressors TIF1α and TIF1β (13, 52, 53). These factors may prevent histone acetylases involved in the relief of repression from gaining access to their substrates.

Binding to a Single ERE Is Sufficient for KRAB Repression—Our studies show that a GAL4-KRAB chimera and an ER-KRAB chimera each exhibit DNA sequence-specific repression and that changing the DNA binding specificity of an ER-KRAB chimera abolishes KRAB repression in ERE-containing genes (Fig. 1). This corroborates earlier findings that tethering the KRAB domain to DNA is required for repression (7–12, 14, 37, 38) and demonstrates that our ER-KRAB chimeras are targeted to EREs.

We find that a single ERE is sufficient for KRAB-mediated repression. After completion of our work, a meeting report described effective repression by a different type of steroid receptor-based KRAB repressor (54). After completion of this paper, successful repression of ERE-containing promoters by ER-NCoR fusions was reported (55). Our data indicate that different rules apply for transcription activation and repression. Although cell type and promoter context play a critical role in the induction of transcription by the ER, the level of KRAB repressor occupancy of the ERE appears to be the overriding factor in repression. In addition, our data demonstrate that it is the presence of the ERE, rather than the capacity for estrogen induction, that determines the potential for repression of a gene by an ER-KRAB chimera. Consistent with these conclusions is our finding that the extent of repression was similar from the thymidine kinase, SV40, and elongation factor 1α promoters containing the same number of EREs, whereas induction by the ER varied from 15-fold to 0 on these promoters.

Interestingly, although synergism between ER bound at different EREs can mask diminished binding (56) when the ER is activating transcription, this is not true for KRAB-mediated repression. Two EREs were clearly more effective in enabling repression by KERK than a single ERE, but there was no further increase in repression in going from two to four EREs (Fig. 6*A*). This contrasts with hER-mediated transcription activation in the same cell line, where strong synergistic effects were seen in comparisons of activity on reporter genes containing one, two, and four EREs (18, 56). Additional support for the idea that tight binding to a response element is important for KRAB repression comes from studies with the promoter fragment containing the pS2 ERE. The ER binds to the pS2 ERE with a lower affinity than to the consensus ERE (15, 41). Despite this diminished binding, hER achieved a 3-fold transcription activation on the pS2 ERE. In striking contrast, KERK was unable to suppress basal promoter activity when bound to the same pS2 ERE. The ability of KERK to partially suppress ER-mediated induction of the reporter containing the pS2 ERE may stem from the ability of KERK to act as a dominant-negative mutant interfering with the binding of wild-type ER, without exerting active repression. In contrast, KERK-3M achieved effective dose-dependent transcription repression of the pS2 ERE. This suggests that high affinity binding to the imperfect ERE, resulting in the continued presence of the ER-KRAB chimera on the promoter, is critical for repression.

Combining Genetic Selection with ER-KRAB Chimeras Provides a Novel Approach to Targeting Genes for Repression—Most studies of gene targeting use multiple rounds of phage display to select mutant DNA-binding domains with affinity for a DNA target (57, 58). The resulting proteins do not provide for ligand-regulated activation or repression. Our surprising finding that binding of ER-KRAB chimeras to the ERE can be modulated either by estrogens or by the widely used SERMs OHT and ICI 182,780 makes ligand-dependent modulation of gene activity feasible using these chimeras. The ability to use the pure antiestrogen ICI 182,780 to activate ER-KRAB repressors enhances their long-term potential for use as gene repressors in breast cancer cells and in other systems in which use of ER agonists would be inappropriate.

We recently described a genetic selection using a modified form of the bacteriophage p22 challenge phage selection system, which requires only a single selection cycle (16). To repress transcription from the imperfect pS2 ERE, it proved

essential to modify the KRAB repressor using information from our recent genetic selection for DBDs with altered and enhanced ERE binding (16). To produce the KERK-3M repressor, we combined information from our genetic selections performed using steroid receptor DNA-binding domains with the KERK chimera, whose ability to repress transcription can easily be modulated using ER ligands. The KERK-3M repressor provides a model for a novel class of gene-targeting protein that combines the ease of use of a ligand-regulated steroid receptor with specificity and affinity gained through large-scale genetic selection. The unique characteristics of these hER-KRAB chimeras make them powerful new tools for the functional analysis of ER-regulated genes.

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Analysis of Estrogen Response Element Binding by Genetically Selected Steroid Receptor DNA Binding Domain Mutants Exhibiting Altered Specificity and Enhanced Affinity*

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To analyze the role of amino acids in the steroid receptor DNA binding domain (DBD) recognition helix in binding of the receptor to the estrogen response element (ERE), we adapted the powerful P22 challenge phage selection system for use with a vertebrate protein. We used the progesterone receptor DNA binding domain and selected for mutants that gained the ability to bind to the ERE. We used a mutagenesis protocol based on degenerate oligonucleotides to create a large and diverse pool of mutants in which 10 nonconsensus amino acids in the DNA recognition helix of the progesterone receptor DNA binding domain were randomly mutated. After a single cycle of modified P22 challenge phage selection, 37 mutant proteins were identified, all of which lost the ability to bind to the progesterone response element. In gel mobility shift assays, approximately 70% of the genetically selected mutants bound to the consensus ERE with a >4-fold higher affinity than the naturally occurring estrogen receptor DBD. In the P-box region of the DNA recognition helix, the selected mutants contained the amino acids found in the wild-type estrogen receptor DBD, as well as other amino acid combinations seen in naturally occurring steroid/nuclear receptors that bind the aGGTCA half-site. We also obtained high affinity DBDs with Trp^{585} as the first amino acid of the P-box, although this is not found in the known steroid/nuclear receptors. In the linker region between the two zinc fingers, G597R was by far the most common mutation. In transient transfections in mammalian cells using promoter interference assays, the mutants displayed enhanced affinity for the ERE. When linked to an activation domain, the transfected mutants activated transcription from ERE-containing reporter genes. We conclude that the P-box amino acids can display considerable variation and that the little studied linker amino acids play an important role in determining affinity for the ERE. This work also demonstrates that the P22 challenge phage genetic selection system, modified for use with a mammalian protein, provides a novel, single cycle selection for steroid/nuclear receptor DBDs with altered specificity and greatly enhanced affinity for their response elements.

Steroid/nuclear receptors (1–4) and many transcription factors belong to protein superfamilies whose members bind to related, but distinct, DNA sequences. Individual proteins within the superfamily must bind to their DNA response elements with high specificity and affinity. The steroid/nuclear receptors bind to a specific DNA sequence, termed a hormone response element (HRE).[1] In general, HREs are composed of two core sequences 5′-AGNNCA-3′ that are separated by a spacer region of 0–6 nucleotides and are arranged as either a direct repeat or an inverted or everted palindrome.

Recognition of HREs by steroid/nuclear receptors is mediated through a DNA binding domain (DBD) of 65–70 amino acids. The core DBD is highly conserved (3). Structural analyses of several DBDs (5–8) showed that they usually contain two independent zinc finger motifs connected by a short flexible amino acid linker, with an amphipathic α-helix near the C terminus of each finger. The first helix in the N-terminal zinc finger, called the DNA recognition helix, is important for specific DNA binding. Upon interaction of the DBD with the HRE, amino acid side chains in the recognition helix make sequence-specific contacts with nucleotides exposed in the major groove of the DNA. A dimerization surface, called the D-box, found in the second helix allows the DBD to recognize the two HRE half sites as a dimer.

Mutational analyses (9, 10) and structural comparisons suggested that the ability of the estrogen receptor (ER), the glucocorticoid receptor (GR), and the progesterone receptor (PR) (5, 6) to discriminate between their respective HREs is at least partially due to three amino acids in the DNA recognition helix of the DBD, called the P-box (11). However, further analysis demonstrated that not all of the side chains of the defined P-box triplet contact the bases of their DNA target. Many contacts involve nucleotides common to both the estrogen re-

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[1] The abbreviations used are: HRE, hormone response element; DBD, DNA binding domain; ER, estrogen receptor; ERE, estrogen response element; GR, glucocorticoid receptor; PR, progesterone receptor; PRE/GRE, progesterone response element/glucocorticoid response element; cERE, consensus estrogen response element; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; WT, wild type.

sponse element (ERE) and the progesterone response element/glucocorticoid response element (PRE/GRE) and involve contacts with the side chains of amino acids conserved in the ER-DBDs and GR-DBDs. Of course, several other factors play a role in DNA binding, including steric hindrance, expulsion of water molecules or ions (12, 13), and alterations of the DNA conformation (13, 14) upon DNA-protein interaction.

The reduced ability to activate transcription of a mutant ER in which the P-box amino acid triplet has been changed to alter binding specificity from the ERE to the PRE/GRE (9) suggests that additional amino acids may play a role in determining affinity for the HRE. We therefore employed discrimination between the ERE and the PRE/GRE as a system for identifying additional amino acids important in binding of a DBD to an HRE.

In the natural process of protein evolution and selection, proteins containing random mutations that confer an advantage on the cell are selected from the large number of neutral or deleterious mutations that occur over time. To simulate the process of natural selection in shifting DNA binding specificity from the ERE to the PRE/GRE, we needed both a system for producing large numbers of mutants with random amino acid changes and a powerful selection for the relatively rare mutant DBDs exhibiting the desired ERE binding properties. We developed a rapid and simple procedure for saturation mutagenesis of a short region of a protein using degenerate oligonucleotides and *Pfu* DNA polymerase. To select the mutants from this large mutant pool that had gained the ability to recognize the ERE, we adapted the powerful P22 challenge phage (15–17) system for use with a vertebrate protein. In the P22 system, substantial numbers of mutants are screened in a single selection cycle using a life-death selection. In this work, we show that the P22 challenge phage selection system can be used to select for mutants exhibiting a substantial change in DNA binding specificity. The P22 challenge phage selection system provides a new tool for engineering steroid/nuclear receptor DBDs with a desired DNA binding specificity and affinity.

To facilitate identification of amino acids important in discrimination between the ERE and the PRE/GRE, it seemed critical to identify the amino acid changes that accompany a shift in DNA binding specificity from the PRE/GRE to the ERE. Because the PR-DBD binds to the PRE/GRE with a higher affinity than the GR-DBD (18), we employed the PR-DBD in these studies.

We selected and identified mutant PR-DBDs containing amino acid sequences exhibiting high affinity binding to the ERE. We find that the first and third P-box amino acids are the most critical residues for DNA binding specificity, and that mutation of amino acids in the linker region can lead to DBDs whose affinity for the ERE is severalfold higher than that exhibited by the wild-type ER-DBD.

EXPERIMENTAL PROCEDURES

Strains—Salmonella typhimurium LT2:MS 1582 carrying P22 $c2^+$ mnt^- prophage MS1868, MS1883 (15) and phage P22 mnt::Kan9 arc(Am)H1065 (15) were used in the challenge phage assays. *Escherichia coli* strain BL21(DE3)pLysS (Novagen, Inc., Madison, WI) was used for protein expression with a vector derived from the pET21b(+) plasmid (Novagen, Inc., Madison, WI).

Plasmid and Phage Constructions—To construct P22 phage carrying the ERE at −3 relative to the transcription start site of the *ant* promoter (Pant), we inserted a double-stranded oligonucleotide containing the consensus ERE (5′-AGGTCAcagTGACCT-3′) into the *Sma*I site of pPY190, which carries a ~500-bp DNA fragment of phage P22 *imm I* DNA cloned into the *Eco*RI–*Hin*dIII sites of pBR322 (15). Plasmid pPY190 containing the ERE was transformed by electroporation into *S. typhimurium* MS1883 (15), and the cells infected by P22 mnt::Kan9arc(Am)H1065 and recombinant phages were selected as a large clear plaque and purified twice on a lawn of MS1582. High titer phage lysates were prepared and purified from MS1883 (15). The presence of the ERE in the P22 phage was confirmed by DNA sequencing.

Plasmid pBAD ER-DBD containing the ER-DBD gene under the control of the arabinose promoter (19) was constructed from pCMVhER by three successive cycles of polymerase chain reaction amplification. This generated a *Sna*BI site followed by a Shine-Delgarno sequence and a unique *Nhe*I site at the 5′-end, and an *Eco*RI site, stop codon, and *Hin*dIII site at the 3′-end. The final product was digested with *Sna*BI and *Hin*dIII and cloned into pBAD18 (19) digested with *Nhe*I and filled in with the Klenow fragment to give a blunt end DNA and consecutively digested with *Hin*dIII.

To prepare plasmid pBAD PR-DBD, the PR-DBD gene from plasmid pGST-PR-DBD (18) was digested with *Nhe*I and *Eco*RI and ligated into pBAD ER-DBD digested with the same enzymes.

Mutagenesis—Saturated random mutagenesis of the PR-DBD recognition helix was carried out by a modified mutagenesis protocol we developed. Two PR-DBD complementary primers were used, with D denoting degenerate nucleotides; 5′-GGTGTCCTTACCTGTDDDDDDTGTAAGDDDTTCTTTAAGAGGDDDDDDDDDDDDDDDDDDDDDDTACTTATGTGCTGGA-3′ and 5′-TCCAGCACATAAGTADDDDDDDDDDDDDDDDDDDDCTTCTTAAAGAADDDCTTACADDDDDDACAGGTAAGGACACC-3′. The nucleotides were randomized with a 17% degeneracy at a 3:3:2:2 ratio of A:C:G:T. The primers were incorporated into plasmid DNA by extension around the plasmid in a 50-μl reaction with 2.5 units of *Pfu* DNA polymerase in 1× *Pfu* buffer (Stratagene), 0.15 fmol of circular plasmid pBAD PR-DBD, and 4.8 pmol of each degenerate primer. The extension reaction was carried out in a thermocycler for 18 cycles with 1 min at 94 °C, 1 min at 50 °C, and 12 min at 68 °C. These extension and amplification conditions differ from those in a recently described *Pfu* mutagenesis protocol (20). The nicked circular DNA products were digested for 1 h at 37 °C in the same buffer with 10 units of *Dpn*I (Stratagene, La Jolla, CA) and directly transformed into *S. typhimurium* host cells by electroporation at 1600 V, 25 microfarads, 200 ohms for screening and selection or into *E. coli* DH5α to prepare DNA for sequencing.

Identification of Specificity Switch Enhanced Affinity Mutants Using Challenge Phage Selection—S. typhimurium MS1868 was transformed by electroporation with the pool of mutated pBAD PR-DBD DNA, plated on LB plates containing 0.2% glucose and 75 μg/ml Timentin (Smith-Kline Beecham, Philadelphia, PA), and incubated overnight at 37 °C. For each challenge phage assay, ~5,000 colonies were pooled and grown in LB liquid media containing 0.2% glucose and 75 μg/ml Timentin to an A_{600} of ~0.6. Bacteria were pelleted and resuspended in LB medium containing 1% arabinose and 75 μg/ml Timentin to an A_{600} of ~0.2. After 1 h, 100 μl of cells were mixed with the P22-ERE phage lysate at a multiplicity of infection of ~25 and incubated at room temperature for 30 min. The infected cells were plated on LB agar containing 1% arabinose, 75 μg/ml Timentin, and 50 μg/ml kanamycin and incubated overnight at 37 °C. Plasmids from lysogens grown on selective medium were purified and sequenced.

Protein Expression and Purification—The T7 expression plasmid pET21PR-DBD, which produces FLAG-PR-DBD, was constructed by cloning the 276-base pair *Nhe*I–*Eco*RI fragment from pBAD PR mutants into the *Nhe*I and *Eco*RI sites of plasmid pET21b(+)ER-DBD (21). Plasmid pET21PR-DBD mutants and pET21ER-DBD were transformed into *E. coli* BL21(DE3)pLys; plated on LB agar containing 0.2% glucose, 34 μg/ml chloramphenicol, and 150 μg/ml ampicillin; and incubated overnight at 37 °C. The bacteria on the plate were pooled and grown in LB liquid medium containing the same concentrations of glucose and antibiotics used on the plates, to an A_{600} of ~0.6. Bacteria were then pelleted and resuspended in LB medium containing 1 mM isopropyl-1-thio-β-D-galactopyranoside and 150 μg/ml ampicillin for 3 h at 37 °C in order to induce protein expression. FLAG-ER-DBD, FLAG-PR-DBD, and the selected FLAG PR-DBD mutants were purified to near homogeneity by immunoaffinity chromatography using the M2 anti-FLAG monoclonal antibody and elution with FLAG peptide (22).

Gel Mobility Shift Assays—Gel mobility shift assays were performed essentially as we have described (21). The reactions were carried out in 20 μl in reaction buffer containing 50 mM KCl, 15 mM Tris-HCl (pH 7.9), 4 mM dithiothreitol, 0.2 mM EDTA, 25 ng of poly(dI-dC), and 10% glycerol. Free probe and protein-DNA complexes were quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Promoter Interference Assays—HepG2 cells were transfected with a total of 8 μg of DNA including 100 ng of the CMV-$(ERE)_2$-CAT promoter interference reporter plasmid (23), the indicated amounts of CMV-FLAG-DBD expression plasmid, 400 ng of pCMV-luciferase (23), and carrier DNA (pTZ18U). 20–24 h after transfection, the cells were subjected to a 3-min shock in 20% glycerol, fed with fresh medium, and

harvested 40–48 h after the glycerol shock. Cell lysates were prepared and assayed for luciferase activity. CAT activity was determined by our mixed phase assay (24).

Transactivation by DBD-VP16 Chimeras—Transfections were performed as described above with some modifications. HepG2 cells were transfected with a total of 8 μg of DNA including 2 μg of $(ERE)_4$-TATA-luciferase reporter plasmid,[2] 40 ng of pRL-Renilla luciferase internal control plasmid (Promega, Madison, WI), one of the pCMV-DBD-VP16 expression plasmids, and pTZ18U as carrier DNA. Dual luciferase assays were performed according to the manufacturer's protocol (Promega, Madison, WI).

RESULTS

The ER-DBD Is Highly Toxic in Bacteria—Both wild-type ER-DBD and high affinity ERE binding mutants expressed in *E. coli* or in the *Salmonella typhimurium* used in the challenge phage assay are highly toxic to the bacteria. Evidence that the ability of the DBDs to bind to ERE sequences was critical to their toxicity came from our observation that ER-DBD mutants, which had lost the ability to bind to the ERE *in vitro*, were not toxic in *E. coli* (data not shown). We concluded that the plasmid loss and cell death that resulted from toxicity of the ERE binding DBDs was due to the presence of nine consensus EREs (cEREs) in the *E. coli* genome (NCBI number U00096). We found that expression from the tightly regulated arabinose promoter (19) minimized toxicity when the inducer was absent. We also replaced the ampicillin in the growth medium with Timentin, a combination of clavulanic acid and ticarcillin, which more effectively blocks growth of bacteria that have lost the expression plasmid and no longer produce β-lactamase.

The Challenge Phage Assay Is a Powerful Assay for Specificity Switch Mutants—Challenge phage are derivatives of bacteriophage P22 that are designed to study protein-nucleic acid interactions *in vivo* (15). The presence of the *imm I* region makes bacteriophage P22 especially well suited to genetic selections based on lysis or lysogeny. The *imm I* region is not present in bacteriophage λ. We use *Salmonella*, not *E. coli*, because P22 cannot infect *E. coli*. Upon infection with a P22 challenge phage, the decision between lysis of the infected *Salmonella* and lysogeny is controlled by expression of the *ant* gene, whose product, the antirepressor (Ant), prevents the establishment and maintenance of lysogeny. Our challenge phage contain a cERE (aGGTCAcagTGACCt) inserted into the *ant* promoter at −3 relative to the transcription start site. We selected for mutant PR-DBDs that bound with high affinity to the ERE. If an infected host cell transformed with the PR-DBD mutant pool does not express a mutant PR-DBD that binds to the ERE, the cell is killed by the P22 phage. If an infected cell expresses a mutant PR-DBD that binds with good affinity to the ERE, the cell survives, because binding of the mutant DBD to the ERE blocks *ant* transcription. In addition, since the challenge phage carry a *Kan9*, cassette, lysogens can be selected as kanamycin- and ampicillin-resistant colonies (Fig. 1). While this system had found significant application, it had not previously been used with a vertebrate protein.

Since our application of the challenge phage selection required a single step selection of mutant proteins exhibiting a substantial change in DNA sequence specificity, in preliminary studies we tested the effectiveness of the selection system. To determine the background of false positive colonies, 10^7 *S. typhimurium* host cells were transformed with different ratios of a control plasmid (pBAD PR-DBD) that produces wild-type PR-DBD, which does not bind to the ERE. The system was spiked with various ratios of the positive plasmid (pBAD ER-DBD) expressing the ER-DBD, which should produce lysogens with the P22-ERE phage. No lysogens were detected when 10^7 cells expressing PR were challenged. When the positive control cells containing the pBAD ER-DBD plasmid were added at a ratio of 1 pBAD ER-DBD:10^6 pBAD PR-DBD cells, five lysogens were obtained. Increasing numbers of lysogens were detected with higher ratios of cells expressing the ER-DBD relative to the PR-DBD (Table I). These data show that the challenge phage selections exhibited an exceptionally low background of <1 false positive lysogen in 10^7 and that a single positive cell can be readily detected in a single selection cycle from a background of 10^6 negative cells.

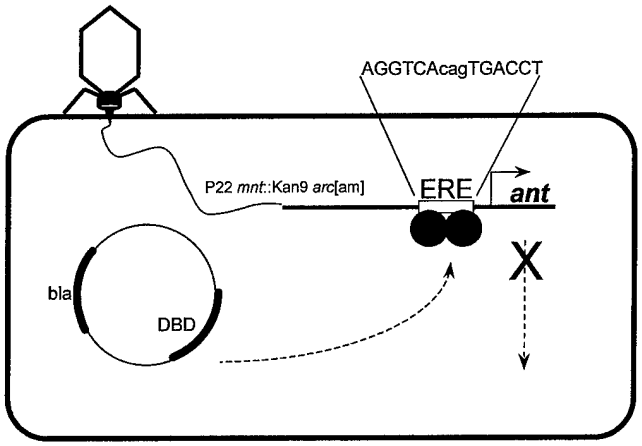

FIG. 1. **Schematic diagram of the challenge phage selection.** A consensus ERE was inserted into the *imm I* regulatory region of the P22 *ant* gene. *S. typhimurium* host cells were transformed with plasmid pools expressing mutated PR-DBDs and challenged with the P22 phage containing the ERE. Binding of a mutant DBD to the ERE inhibits *ant* gene expression, leading to formation of viable lysogens. To prevent formation of colonies by bacteria, which do not contain the phage, the *mnt* gene in the bacteriophage P22 has been replaced with a constitutively expressed kanamycin resistance gene, allowing selection for lysogens resistant to kanamycin and Timentin.

Mutagenesis of the PR-DBD—To study the roles of amino acids in the recognition helix in ERE binding, we had to consider the strong selective pressure against changes in the zinc finger region of the DBD (26). To avoid generating a pool in which almost all of the mutants had lost their ability to bind to the ERE, we created a pool of mutants that averaged three mutations per protein. We did not mutate the amino acids conserved in the ER and PR-DBDs, which are known to be important for maintaining the structure and function of the protein (amino acids 587–588 and 590–593 in PR). We randomly mutated the nonconsensus amino acids shown in boldface type ^{585}**GSCKVFFKRAMEGQHN**^{600} in the PR-DBD recognition helix (Ref. 27; Table II). We tried several mutagenesis methods including error-prone polymerase chain reaction (28, 29). The only mutagenesis method to achieve the requisite density and localization of mutations was a protocol we developed using *Pfu* DNA polymerase. In this protocol, a pool of oligonucleotides, degenerate in the region of interest and containing complementary sequence encoding the wild-type sequence at both ends, was prepared and annealed to the wild-type pBAD PR-DBD, and the mutations were incorporated by extension with *Pfu* DNA polymerase (see "Experimental Procedures"). Parental wild-type plasmids were eliminated by digestion with *Dpn*I. The mutant pool was transformed directly into bacterial cells without ligation, resulting in a library size of approximately 10^9 per set of degenerate oligonucleotide primers. Sequencing individual mutants prior to selection dem-

[2] G. De Haan, S. Chusacultanachai, and D. J. Shapiro, submitted for publication.

TABLE I
The challenge phage assay can identify one mutant in >10^6 negative cells

Positive cells with ER-DBD:Negative cells with PR-DBD[a]	Number of lysogens
0:10^7	0
1:10^6	5
1:10^5	33
1:10^4	212
1:10^3	1244

[a] 10^7 cells containing different ratios of positive control plasmid (pBADER-DBD) and negative control plasmid (pBADPR-DBD) were challenged with P22-ERE phage as described under "Experimental Procedures."

onstrated that they contained the expected distribution and frequency of random mutations (data not shown). This mutagenesis strategy provides a rapid, simple, and effective way to create a highly saturated mutant library with a controlled mutation rate.

Selection of PR-DBD Specificity Switch Mutants and Identification of Mutated Amino Acids—In each screen used to identify the specificity switch mutants ~5,000 independent transformed cells were pooled and plated, DBD expression was induced with arabinose, and the cells were infected with the P22-ERE phage. Ten million induced cells were challenged with the ERE phage and plated on selective medium. 50 out of 100 plates of mutants screened produced lysogens (20–1026 colonies/plate). One lysogen was selected from each positive plate for further analysis by DNA sequencing. We obtained 37 independent mutants containing an average of five mutated amino acids (Table II).

Sequence analysis of all 37 genetically selected specificity switch mutants showed that all of the nonconserved amino acids and the P-box amino acids were mutated with high frequency (Table II). In the P-box, which is GSV in the PR-DBD and EGA in the ER-DBD, Gly^{585} and Val^{589} were mutated with 97 and 92% frequency, respectively. Gly^{585} was mutated either to Trp or to Glu, the amino acid in the ER-DBD. Ser^{586} was unchanged in 40% of the mutants and was mutated to Gly in the remaining 60%. Val^{589} was mutated to Ala, Gly, and Ser with 68, 25, and 7% frequency, respectively. While there is no single amino acid mutation in the linker region common to most of the mutants, changes to basic amino acids occurred with a high frequency, and the mutation G597R was present in 10 of the mutants.

Characterization of the Specificity Switch Mutants—The 37 selected mutant DBDs were subcloned into the FLAG expression system, expressed as FLAG epitope-tagged proteins, and purified by immunoaffinity chromatography with anti-FLAG monoclonal antibody. The affinity of each of the mutant DBDs for the cERE, for the imperfect pS2 ERE (5′-aGGTCAnnnTG*G*CCc-3′), and for the PRE/GRE was compared with that of the ER-DBD and the PR-DBD in protein titrations using quantitative gel mobility shift assays (Fig. 2, *A* and *B*). Relative affinity for the consensus ERE was determined from the concentration of protein required to upshift 50% of the probe. In agreement with our earlier work (21), wild-type ER-DBD showed little or no detectable binding to the imperfect pS2 ERE in gel shift assays. In contrast, the genetically selected mutants displayed high affinity binding to the pS2 ERE (Fig. 2*B*). While the starting PR-DBD effectively bound to the PRE/GRE (5′-AGAACAnnnTCTTGT-3′) and we carried out only a positive selection for binding to the cERE (5′-aGGTCAnnnTGACCt-3′), all 37 of the selected mutants completely lost the ability to bind to the PRE/GRE (Fig. 2*C* and Table II). This indicates that high affinity binding to one DNA recognition sequence is incompatible with binding to a different recognition sequence. Mutants selected using the P22 challenge phage system are therefore highly specific for binding to the DNA sequences of interest.

In gel shift assays, 17 of the 37 mutants exhibited 10–15-fold higher affinity for the cERE than wild-type ER-DBD. 14 of the mutants exhibited 2–9-fold higher affinity binding to the cERE than was shown by the ER-DBD. Two of the mutants bound to the cERE with an affinity lower than the ER-DBD, and four mutants showed no detectable binding to the ERE or to the PRE/GRE (Table II). Whether these four mutants bind to the ERE with an affinity below the threshold of detection in our gel shift assays or are false positives was not examined. Evidence suggesting that these mutants may bind weakly to the ERE and are not random false positives comes from the observation that all four of the nonbinders contained mutations that changed one of the three amino acids in the P-box of the PR-DBD to the corresponding amino acid in the ER-DBD P-box. In contrast, in all 33 of the mutants exhibiting binding to the cERE in gel shift assays, at least two of the three critical amino acids in the PR-DBD P-box were mutated.

Mutations in the Linker Amino Acids Enhance Affinity for the ERE—Surprisingly, high affinity binding to the ERE by the selected mutants was associated with mutations in the linker region (amino acids 594–600). In the structures of steroid hormone receptor DBDs, this region is rather poorly ordered and forms a flexible linker between the first and second zinc fingers (5, 6). Consistent with the importance of flexibility in this region, mutations to Pro were relatively common. The most striking mutation was G597R, which was present in 10 of the 23 mutants exhibiting >7-fold higher affinity for the ERE than wild-type ER-DBD. In contrast, none of the six selected mutants whose affinity for the ERE was lower than that of the wild-type ER-DBD contained the G579R mutation. Since 14 of the 17 mutants exhibiting a >9-fold increase in binding relative to the ER-DBD contain at least one linker region mutation to a basic amino acid, mutations to basic amino acids are clearly important. While these positively charged residues probably exhibit electrostatic interactions with the negatively charged phosphate backbone, they appear to increase affinity for the DNA without decreasing the specificity of ERE recognition. The importance of ionic interactions is illustrated by comparing mutants 50 and 56 (Table II), which contain the same mutations in the P-box amino acids. Mutant 50, with an affinity for the ERE 12-fold higher than wild-type ER-DBD has an M595K mutation, while mutant 56 with an affinity for the ERE 10 times lower than ER-DBD has a Q598D mutation. Mutations to Asp were rare in the proteins exhibiting high affinity binding to the cERE and were present in both mutants exhibiting reduced binding to the cERE.

Mutations to nonpolar amino acids containing aliphatic side chains were also common in the high affinity binders. Several amino acids (Cys, Met, Phe, and Trp) present at low abundance (1–3%) in proteins were also rarely seen in the genetically selected mutants. Since our mutagenesis was random, their absence in the selected mutants suggests that their presence imposes structural or folding constraints on the DBD.

Mutants Exhibiting High Affinity Binding to the Consensus ERE Also Bind to the Imperfect pS2 ERE with High Affinity—Imperfect EREs, not the cERE, are found in almost all ERE-containing genes. Both the full-length ER and the ER-DBD exhibit reduced affinity for these imperfect EREs (30). We tested the ability of the mutants to bind to the imperfect ERE, found in the estrogen-inducible human pS2 gene (31). The wild-type ER-DBD exhibits extremely weak binding to the pS2 ERE (Ref. 21; Fig. 2*C* and Table II). The highest affinity mutants bound to the pS2 ERE with >1000-fold higher affinity

TABLE II

Relative binding affinity of the PR-DBD specificity switch mutants for the concensus ERE, PRE/GRE, and pS2 ERE

Mutant		Relative binding affinity[a]		
Number	Amino acid sequence	Consensus ERE	PRE/GRE	pS2 ERE
	EGCKAFFKRSIQGHND (WT ER)	100	0	1
	GSCKV**F**FKR**AMEGQHN**[b] (WT PR)	0	100	0
41	EG A N PRI	1500	0	>1000
42	WG A NRH S	1500	0	>1000
57	W G K VPT	1500	0	>1000
55	WG A VRPT	1500	0	>1000
5	W A IA G	1500	0	>1000
15	E A G H Y	1300	0	>1000
7	WG S AYR S	1300	0	>1000
29	WG G SRK	1300	0	>1000
31	EG A R PR	1300	0	>1000
50	W G K	1200	0	>1000
52	EG A SNR Q	1000	0	>100
25	W G KDVH	1000	0	>100
27	WG A KA S	1000	0	>100
14	W G LKR	1000	0	>100
4	EG A ARRL	1000	0	>100
49	W G IT KK	1000	0	>100
45	EG A I	1000	0	>100
53	W G D Y	900	0	>100
12	EG A ARR	900	0	>100
48	EG A SR REI	800	0	>50
59	EG A R R Q	800	0	>50
47	EG A I NA	800	0	>50
22	WG A ST T	700	0	>50
13	EG A YG	500	0	>50
51	W G N YH	500	0	>10
9	EG A SKD H	500	0	>10
2	WG A R	300	0	>10
37	EG A S H	300	0	>10
54	W A PY	200	0	
17	EG A R	200	0	
26	EG A	200	0	
40	EG A D G	50	0	
56	W G D	10	0	
16	E G HNK			
18	E Q SL S			
19	E L TS			
24	A W			

[a] Binding of WT ER-DBD to the consensus ERE and of the WT PR-DBD to the PRE/GRE were set equal to 100. Estimated binding of the WT ER-DBD to the pS2 ERE was set to 1. Binding affinity was calculated from the amount of purified DBD required to shift 50% of the labeled probe in gel mobility shift assays. Relative binding is the ratio of binding by the mutant to binding by WT protein.

[b] The mutated amino acids in the PR-DBD are shown in boldface type.

than the wild-type ER-DBD (Fig. 2*C* and Table II). The mutant's ability to bind to the imperfect ERE was particularly striking, since even the highest affinity mutants retained specificity for ERE binding and showed no binding at all to the PRE/GRE.

The Mutants Selected in Bacteria Exhibit High Affinity Interaction with the ERE in Mammalian Cells—Since the mutants were identified by genetic selection in bacteria and assayed for ERE binding *in vitro*, it was important to evaluate the ability of a few of the mutants to function in mammalian cells. To more directly evaluate the ability of the mutants to bind to the ERE *in vivo*, we carried out promoter interference assays (23). In these assays, mutants bound to EREs near the initiation site of the CMV promoter compete for binding with basal transcription factors. The amount of transfected expression plasmid required to produce a given level of interference with transcription provides a measure of the interaction of the expressed protein with the ERE. We transfected increasing amounts of DNA encoding mutant DBDs into HepG2, human hepatoma cells, and determined the extent of promoter interference for each DNA. The control PR-DBD did not inhibit transcription. All three tested mutants were clearly more effective in interfering with the activity of the CMV-$(cERE)_2$-CAT promoter than the wild-type ER-DBD (Fig. 3). Mutant 26, with an affinity for the consensus ERE twice that of the wild-type ER-DBD was only slightly more effective than the wild-type ER-DBD. Mutants 5 and 15, with affinities for the ERE 15- and 13-fold higher than wild-type ER-DBD, respectively, required 5–20-fold less transfected DNA to achieve 40% inhibition of promoter activity than the ER-DBD. These data demonstrate that the selected mutants bind to the ERE in intact human cells with far higher affinity than the wild-type ER-DBD.

To analyze the ability of the mutants to activate transcription, we fused the strong VP16 transactivation domain (32) to each of the mutants and to the ER and PR-DBDs, and we expressed the chimeric proteins from the CMV promoter. HepG2 cells were cotransfected with a range of concentrations of each of the chimeric proteins and an ERE-containing luciferase reporter gene. The control PR-DBD-VP16 was unable to activate the reporter gene, while the wild-type ER-DBD-VP16 elicited detectable transactivation only at the highest level of transfected DNA, 25 ng. All three of the mutants exhibited higher levels of transactivation than the WT ER-DBD-VP16. Transactivation by the mutants was related to their affinity for the ERE. Mutant 26 was the least effective, while mutant 5 was slightly more potent than mutant 15 (Fig. 4). Similar results were obtained using a reporter gene containing a single ERE (data not shown).

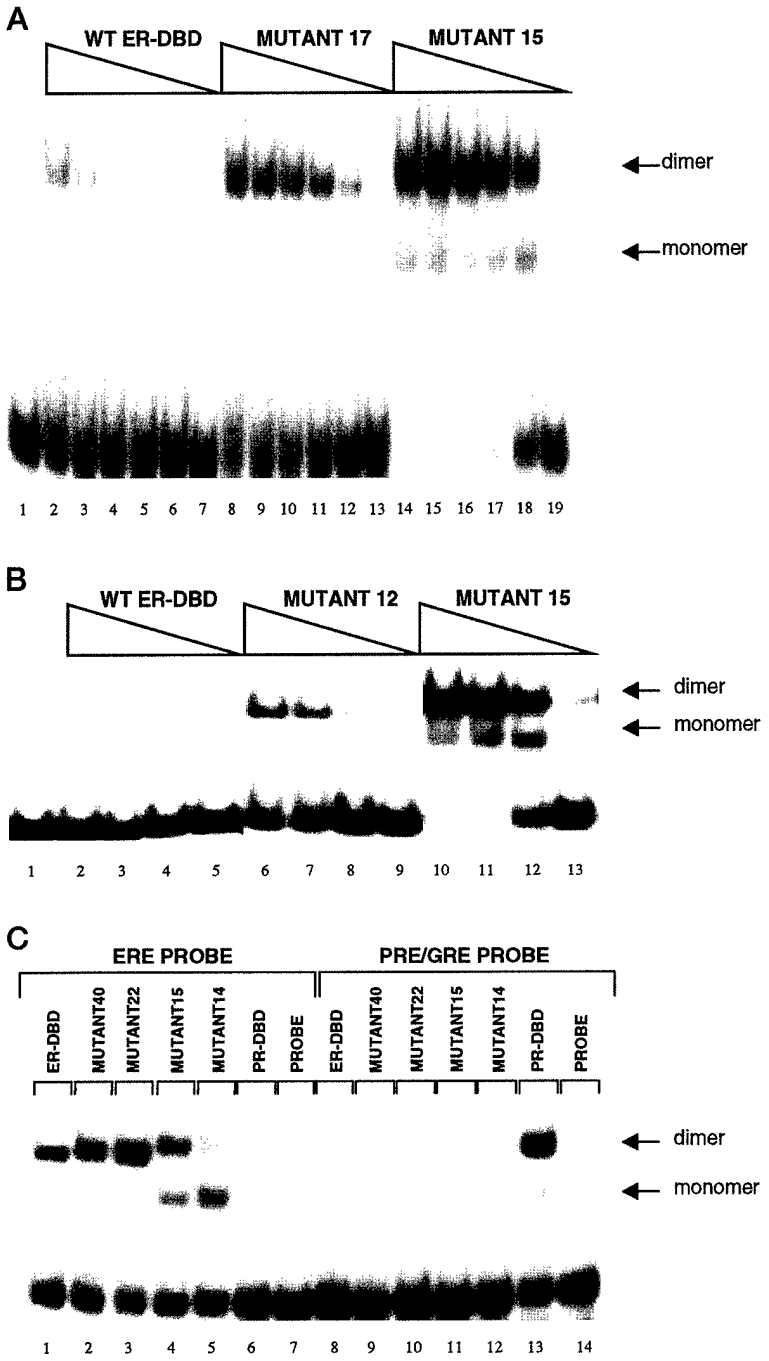

FIG. 2. **Characterization of the specificity switch mutants in gel mobility shift assays.** Gel shift assays were carried out as described under "Experimental Procedures." *A*, increasing amounts of purified WT ER-DBD (*lanes 2–7*), mutant 17 (*lanes 8–13*), and mutant 15 (*lanes 14–19*) were incubated with the consensus ERE probe. The concentrations of protein were 5 (*lanes 7*, *13*, and *19*), 10 (*lanes 6*, *12*, and *18*), 25 (*lanes 5*, *11*, and *17*), 50 (*lanes 4*, *10*, and *16*), 100 (*lanes 3*, *9*, and *15*), and 200 nM (*lanes 2*, *8*, and *14*). *Lane 1* contained ERE probe alone. Although the affinity of the wild-type ER-DBD for the ERE is too low for it to bind as a monomer to an ERE half-site in gel shift assays (Ref. 18, and *lanes 2–7*), mutant 15 and several other high affinity mutants showed clearly detectable monomer binding to the ERE. *B*, increasing amounts of ER-DBD (*lanes 2–5*), mutant 22 (*lanes 6–9*), and mutant 15 (*lanes 10–13*) were incubated with the pS2 ERE probe. The concentrations of protein were 5 (*lanes 5*, *9*, and *13*), 50 (*lanes 4*, *8*, and *12*), 100 (*lanes 3*, *7*, and *11*), and 500 nM (*lanes 2*, *6*, and *10*). *Lane 1* contained pS2 ERE probe alone. *C*, ER-DBD (*lanes 1* and *8*), mutant 40 (*lanes 2* and *9*), mutant 22 (*lanes 3* and *10*), mutant 15 (*lanes 4* and *11*), mutant 14 (*lanes 5* and *12*), and PR-DBD (*lanes 6* and *13*) were incubated with either labeled cERE probe (*lanes 1–6*) or labeled PRE/GRE probe (*lanes 8–13*). *Lanes 7* and *14* contained probe alone (ERE and PRE, respectively).

DISCUSSION

Mutation of Amino Acids in the P-box Is Necessary for Altering HRE Specificity but Is Insufficient for High Affinity Binding to the ERE—Previous studies showed that mutating three amino acids in the P-box (amino acids 585–589) from GSckV found in the PR-DBDs and GR-DBDs to EGckA, which is found in the ER-DBD, was critical to the ability of a DBD to discriminate between the PRE/GRE and the ERE (9, 10). The introduction of amino acid substitutions in the P-box, one amino acid at a time, has also been reported (33–37). We used random mutagenesis to simultaneously mutate the P-box amino acid triplet and the previously unstudied linker region and used a powerful genetic selection to isolate mutants that had gained the ability to bind to the ERE. Our data indicate that there is some flexibility in both the number and nature of the P-box mutations.

Of the 17 selected mutants exhibiting >9-fold higher binding to the ERE than the wild-type ER-DBD, seven retained the Ser at the second position of the P-box (amino acid 586) seen in the PR, and 10 contained the Gly found in the ER. Although the second amino acid in the P-box appears to play a very limited role in discrimination between different HREs, there are rigid requirements for Ser or Gly at this site. All 37 selected mutants contain either Ser or Gly at this position. The first and third amino acids in the P-box (Gly^{585} and Val^{589} in the PR) are the most critical residues for HRE recognition. Since none of the selected mutants that bind to the ERE *in vitro* exhibit changes in only one of these amino acids, we conclude that Gly^{585} and Val^{589} must both be mutated for effective ERE binding. While the spectrum of amino acids tolerated at these positions is quite limited, a unique set of amino acids is not required. The amino acids we observed at Val^{589} of the P-box (Ala, Gly, and Ser) are

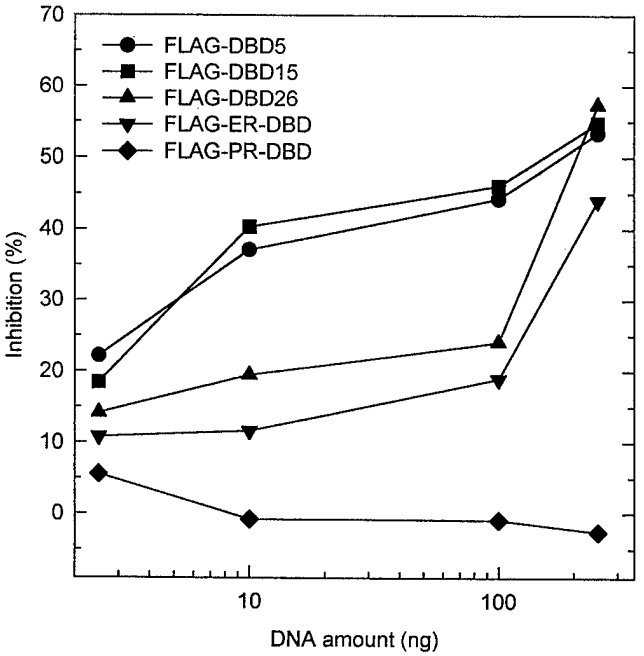

FIG. 3. **The mutants exhibit increased binding to the ERE in human cells.** HepG2 cells were transfected with 100 ng of the promoter interference reporter plasmid CMV-$(ERE)_2$-CAT and increasing amounts (1, 10, 100, and 500 ng) of the expression plasmid encoding FLAG-DBD5 (●), FLAG-DBD15 (■), FLAG-DBD26 (▲), FLAG-ER-DBD (▼), and FLAG-PR-DBD (♦). CAT activity in the absence of DBD expression plasmid was set equal to 100%, and the percentage of inhibition of CAT activity was determined for each mutant. Each point represents the average of at least two separate transfections.

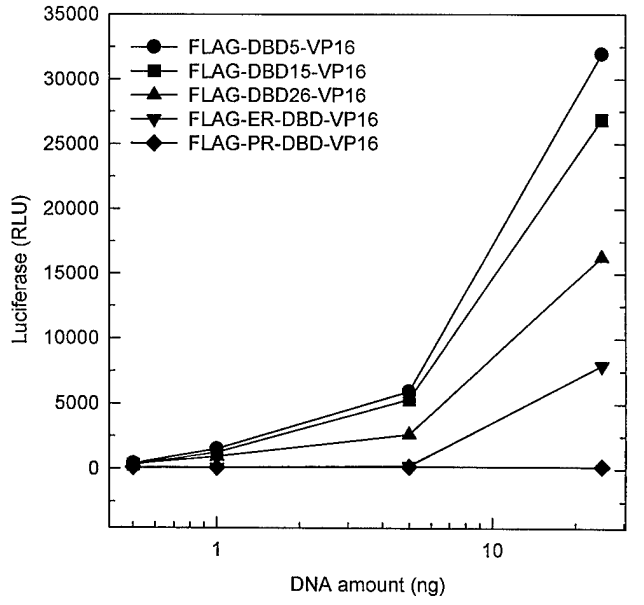

FIG. 4. **The mutants exhibit enhanced transactivation ability in human cells.** HepG2 cells were transfected with 2 μg of 4ERE-TATA-luciferase reporter plasmid and increasing amounts of FLAG-DBD5-VP16 (●), FLAG-DBD15-VP16 (■), FLAG-DBD 26-VP16 (▲), FLAG-ER-DBD-VP16 (▼), or FLAG-PR-DBD-VP16 (♦). The data for each point represent the average from at least two separate transfections. *RLU*, relative luciferase units.

all present in known members of the steroid/nuclear receptor superfamily that recognize the ERE half-site.

In the ER and in most steroid/nuclear receptors that recognize the ERE half site, Glu is present at the first position in the P-box. G585E was present at this position in about half of the mutants, and G585W, which is not found at this position in any member of the steroid/nuclear receptor superfamily, was present in the other half of the mutants. Of the 17 mutants whose affinity for the ERE was >9-fold higher than that of wild-type ER-DBD, 11 contained G585W, and only six contained G585E. In a previous study in which this Gly in the GR-DBD was mutated to Trp, this change resulted in promiscuous binding to many response elements including the PRE/GRE and the ERE (33–34). Although there was no genetic selection against PRE/GRE binding in our study, all of the selected mutant proteins containing G585W showed no detectable binding to the PRE/GRE in both protein titration gel mobility shift assays (Fig. 2*C* and Table II) and in competition gel mobility shift assays performed with a 200-fold excess of unlabeled PRE/GRE (data not shown). This high specificity for ERE binding may result from the presence of multiple amino acid mutations in the recognition helix of our mutants. Mutants with Trp at the first P-box position also bind to the ERE *in vivo* in promoter interference assays using mutant number 5 (Fig. 3), and effectively activated transcription when linked to the VP16 activation domain (Fig. 4).

While changing the P-box amino acids was essential for altering specificity from binding to the PRE/GRE to the ERE, it was insufficient for high affinity binding to the ERE. Mutant number 26, in which the only changes are to the *EG*ck*A* sequence seen in the ER, exhibited a lower affinity for the ERE than 75% of the selected mutants.

Mutations in the Linker Region of the DBD Result in Strongly Enhanced Binding to the ERE—The linker region between the two zinc fingers in the ER-DBD is identical in almost all species (38). In the crystal structures of steroid receptor DBDs, the linker appears to be flexible, without higher order structure, and is in close proximity to the phosphate backbone of the DNA helix. Mutation of amino acids in this region dramatically increases binding to the ERE. Amino acids with basic side chains, like Lys and Arg are associated with high affinity mutants, whereas amino acids with acidic side chains are found primarily in the mutants exhibiting reduced or undetectable binding to the ERE (Table II). Despite the prevalence of basic amino acid substitutions in this region, lysine and arginine were not always interchangeable. While G597R was present in 10 of the 22 mutants exhibiting >7-fold higher affinity for the ERE than wild-type ER-DBD, G597K was absent. Mutations to amino acids with aliphatic side chains and to tyrosine with its phenolic hydroxyl group were also commonly found in the high affinity DBDs. These side chains may contact the sugar ring of the DNA backbone (8) and help stabilize the protein-DNA complex.

Imperfect EREs often contain a consensus half-site and a nonconsensus half-site, which differs from the consensus half-site by 1–3 nucleotides. The ER-DBD recognizes these imperfect sequences by low affinity binding using an alternative side chain conformation (39). Most of the selected mutants exhibited a 50–1000-fold higher affinity for the imperfect ERE found in the pS2 gene than the wild-type ER-DBD. We believe this dramatic increase in binding relative to ER-DBD is due to a combination of the higher affinity for the ERE half-site of the selected mutants and to the presence of a robust dimerization interface in the PR-DBD (6). Many of the mutants with a high affinity for the ERE were able to bind to the ERE as a monomer (Fig. 2*A*) and will effectively occupy the consensus ERE half-site in the pS2 ERE. The formation of a dimerization interface on the DNA facilitates binding of the mutants to the imperfect pS2 half-site. When a mutant dissociates from its low affinity binding site on the imperfect pS2 half site, it remains tethered to the DNA through the strong dimerization interface, and its high local concentration strongly facilitates rebinding to the

imperfect half site. This combination of enhanced affinity for the consensus half-site and dimerization to facilitate rebinding to the low affinity imperfect half-site is probably responsible for efficient binding of the selected mutants to the pS2 ERE. Our observation that several of the mutants have gained the ability to bind to the ERE as monomers and the strong bias of the mutations toward basic amino acids strongly support the view that enhanced binding is a result of direct interaction between the mutated region of the DBD and the DNA. However, it remains possible that some of the mutants exhibit enhanced dimerization. In a study in which the P-box amino acids were mutated, protein-protein interactions appeared to make a major contribution to the ability of T3Rβ-RXRα heterodimers to bind to HREs (37).

Production of Specificity-shifted Enhanced Affinity DNA Binding Proteins Using the P22 Challenge Phage System—Production of recombinant proteins targeted to a DNA sequence of interest requires methods for producing large pools of mutants and a powerful selection technique to identify and isolate the mutants of interest.

We found that available mutagenesis methods were unsuitable for saturation mutagenesis of a defined segment of a protein, such as the DNA recognition helix. We therefore developed a simple rapid mutagenesis method using doped oligonucleotides and *Pfu* DNA polymerase. The use of degenerate oligonucleotides allows precise delineation of the amino acids to be mutated and permits retention of amino acids important in protein function. Because doped oligonucleotides are used and the nucleotide ratios can be adjusted, true random mutagenesis is readily obtained. Using *Pfu* DNA polymerase, under the conditions we describe, allows production of large mutant pools without isolation of DNA or ligation, steps that often limit the number of independent sequences in mutant pools.

While the P22 selection system had been used in a number of prokaryotic systems, it had not been applied to a vertebrate protein and had not previously been used to isolate proteins exhibiting far higher affinity for a DNA sequence than the naturally occurring protein that recognizes the site. Instead, most efforts to isolate mutant proteins with defined DNA sequence specificity have focused on the use of selection strategies based on phage display (40–42). Despite its unquestioned utility, the number of false positives generated and the relatively low signal:noise ratio of phage display almost always makes it necessary to perform multiple cycles of selection. In contrast, we show that the P22 challenge phage system can be used to identify one positive cell in a million cells in a single selection cycle (Table I).

The tailless subfamily of orphan receptors carries Asp at the first position of the P-box (25) and binds to an 5′-AAGTCA-3′ half-site that differs from the consensus ERE half-site used in our selections by one nucleotide (5′-AGGTCA-3′). The impressive DNA sequence selectivity of the challenge phage selection system is illustrated by the fact that none of the 37 mutant DBDs we isolated and characterized contained Asp at the first position of the P-box. The high sequence selectivity of the challenge phage system may be related to its use of *in vivo* selection in the presence of the bacterial chromosome. Since the bacterial DNA is present in great excess over the target sequence, it serves as a nonspecific competitor DNA during the *in vivo* selection.

In this work we describe modified conditions for using the bacteriophage P22 challenge phage selection system with a toxic vertebrate protein and demonstrate the feasibility of using this selection system to generate DNA-binding proteins with altered sequence specificity and greatly enhanced affinity for a recognition sequence. This system should prove useful in studying other protein-DNA interactions and for engineering proteins with novel DNA binding specificity. After fusion to activation, repression, or catalytic domains, these engineered DNA binding modules can have a variety of potential regulatory and therapeutic applications.

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